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An epidemiological and virological study of influenza

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AN EPIDEMIOLOGICAL AND VIROLOGICAL STUDY OF INFLUENZA

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS Mr J. M. VAN BEMMELEN, HOOGLERAAR IN DE FACULTEIT DER RECHTSGELEERDHEID, TEGEN DE BEDENKINGEN VAN DE FACULTEIT DER GENEESKUNDE TE VERDEDIGEN
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AAN ANN
LODEWIJK
DIRK
ERIK
FRANKLIN

TER VERANTWOORDING

Slechts zelden is men in de gelegenheid hen te bedanken, die bijgedragen hebben tot de vorming van het eigen ik. Zelden ook is men in staat zich te verantwoorden over de keuze van een bepaald werk en over de wijze van uitvoering daarvan. Ik neem daarom met plezier de gelegenheid te baat, voor zover mogelijk, mijn dank te betuigen aan hen, onder wier invloed ik mijzelf geworden ben en die mij in staat gesteld hebben dit werk ten uitvoer te brengen.

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Naderhand ben ik gaan beseffen wat mij zomaar in de schoot geworpen werd door het contact met de vele bijzondere mensen die bij ons thuis kwamen. Het is niet mogelijk een ieder afzonderlijk te noemen. De vrienden van mijn ouders uit Amsterdam en later uit Utrecht, zij allen hebben bijgedragen tot mijn vorming.

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wetenschappelijk werk wel ieder uit zijn omgeving moest infecteren.

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Met het verschijnen van dit proefschrift besef ik zeer goed geen „meesterstuk" geleverd te hebben. Toch is het bedoeld als proeve van mijn bekwaamheid. Ik heb getracht de verschillende kanten van één probleem: de epidemiologie van virusziekten, met influenza als model, te belichten. Daarvoor was het nodig tot een probleemstelling te komen, methodes van onderzoek te ontwikkelen en op hun waarde te toetsen, epidemiologisch veldwerk te verrichten en uit te werken en bovendien nieuwe problemen, die zich tijdens het onderzoek hadden voorgedaan te stellen en te bewerken. Tenslotte heb ik ook een voorbeeld van „speculatief" wetenschappelijk onderzoek zonder direct aanwijsbaar „nut" bewerkt. Het heeft mij moeite gekost aan de eis voor de wetenschappelijk werker, zoals Sir Almroth Wright deze destijds formuleerde: „Work, finish, publish" te voldoen.

CHAPTER I

Introduction

Although the clinical symptomatology of a number of contagious diseases was known and described long before the beginning of our era, it remains difficult to deduce from old manuscripts whether there was in fact more than the most rudimentary understanding of the causes. Antiquity must have had some notion of the transmission of diseases from person to person it is open to question whether the fear with which these diseases were regarded arose from the mysterious aura that was woven around them or whether the danger of contagion was also recognized. It is known that the fear of contagion played a considerable part later on and that all kinds of measures were devised to render the chance of contagion as slight as possible. Though waning supernatural influence had not ceased altogether by the middle ages, but it is generally assumed that masks and other special accoutrements were directed more against the danger of contagion than for the conjuring of diseases. This is also clear from the measures taken in the field of isolation and quarantine, which were already known and applied in the late Middle Ages.

In studying early medical literature it strikes one how much had already been known for quite a long time. The infectious element was still an unknown concept, and they spoke of a *genius epidemicus*. To this they ascribed properties which hold good even now: virulence, mitigation etc.

However, much has been changed since the discovery of micro-organisms as the causative agents. Besides studying the symptoms of the disease in man it is possible now to examine pathogenic germs.

We owe the great progress achieved in epidemiology to our ability to become acquainted with the peculiarities of the "virus" as distinct from clinical symptoms.

It became possible to assemble valuable data, especially of the diseases caused by bacteria. We do not know, however, to what degree the results of bacteriological investigation can be applied to filtrable viruses. Among the problems posed in the study of the epidemiology of an infectious disease appear the following:

a) how is the infection transmitted from one person to another?
b) whether every infected person exhibits morbid symptoms;
c) whether apparently healthy people help the disease to spread (virus carriers);

d) what is the reason why some people who have been in close contact with the disease do not fall ill?
e) whether fluctuations in the seriousness of the syndrome may also be caused by variations in the virus;
f) whether suffering once from a disease renders a person inmmune to further attacks of the same virus;
g) whether other micro-organisms play a part in the course of the epidemic.

Briefly, one may say that there are two aspects of the problem of epidemiology — the virus and its victims — and that they interact on each other. For this reason it is not possible to study the one without the other.

I have considered the filtrable viruses to decide which of them lends itself best to epidemiologic examination. It would have to be easy to work on, but at the same time, the disease caused by it would have to be one occurring naturally in our own environment. In this respect the influenza virus is a good example. In consequence of the work done all over the world with this virus a good deal of data are known and the examination methods concerned are easily assimilated by those who are to conduct the research. But these are not the only factors affecting the selection of a virus. Were they in fact so, I could have chosen the mumps virus. Technically it is as easy to study as the influenza virus. The number of serologic reactions described for it is even greater than that for influenza. However, influenza has the great advantage that experiments can be carried out on easily obtainable laboratory animals. Moreover mumps exhibits a quite different epidemiological pattern—one infection with parotitis epidemica nearly always confers lifelong immunity. Its choice therefore would exclude a number of vital problems to be studied. Hence, we have chosen influenza as the subject of our epidemiologic examination. Thanks to the cooperation of the Director and physicians of Endegeest Asylum, we were given the opportunity of studying an influenza epidemic that occurred there during the winter of 1948.

In the first place we investigated the reaction of man to the infection. Serologic investigation was aimed at answering these questions:

1. What was the degree of immunity of the population to be examined, before the epidemic occurred.
2. Whether, and to what extent, antibodies were produced under the influence of the infection by
   a. persons with clinical symptoms
   b. others.

3. How, and to what extent, the immunity situation of the population had been affected by the epidemic (expressed as a change in the quantity of circulating antibodies).

Concerning items 1 and 3 it was sufficient for our purposes to gather serum specimens before and after the epidemic. It was easy, also, to lay down lines of action for the examination of persons with clinical symptoms. Immediately after the disease was reported we took blood samples from patients found to be in the acute phase of the disease, and again between 10 and 14 days afterwards. In studying those without influenza symptoms we were in the dark as regards the moment of infection, if any.

The investigation would have been simpler if there had been an infectious explosion. Then we could have drawn off blood from all the persons involved in the examination at that moment and we could have repeated it after 3 or 4 weeks. However, when it became clear that the epidemic was of a more lingering character, this plan proved insuitable. It would have been preferable if blood could have been taken from the patients weekly or even more often. We did consider this but realised that it was impossible. The inconvenience to patients, physicians and the nursing staff of the asylum deterred us from proposing this course, and the enormous amount of work compared with the slight chance of important results clinched the matter. At this stage one feels the need of a reliable micromethod for serological research. True enough, the Fulton (18) method of micro complement fixation may be performed with very little material, but its laboriousness excludes it for large scale application. In the second chapter a method we have developed will be described. Necessity was the mother of this particular invention, but its birth occurred too late to be of use in this study.

At first we were afraid the clinical diagnosis of "influenza" would prove unreliable, but during the course of the epidemic it appeared that our fears were groundless. The reports of the clinician who made the diagnosis on the basis of the symptomatology given by Mulder et al., tallied remarkably well with our results. For the rest I have ignored clinical considerations, not being competent in this field.

To turn now from the victim to the virus, in the first place the question of its demonstration and isolation arises, followed by the examination and comparison of the isolated strains.

Finally the behaviour of the *genius-epidemicus* during the epidemic has to be observed.

It is possible to transmit the influenza virus directly from man to the extra-embryonal cavity of incubated hens eggs or to laboratory

animals (ferrets, hamsters). These methods of investigation have their drawbacks: the extensive work to be done, high cost and the large quantity of material required. It would be necessary to collect material regularly — preferably daily — from all persons involved in the examination and to investigate whether it contains virus, the object being to learn something about the part played by "virus carriers" during an epidemic. Technically as well as financially this was not possible, and we conducted a search for new methods to get results in a cheaper and simpler way. We hoped to be able to develop a kind of "screening-test" for tracing the virus in garglings. I will return to this subject in the next chapter, where I will explain why I did not succeed in finding an entirely satisfactory solution to the problem, and why we had to give up our pursuit of virus carriers.

The comparative examination of the strains isolated by us during the epidemic is a problem in itself. Some research-workers draw certain conclusions about the behaviour of the virus *in vivo* from the variation in characteristics they find. They are of the opinion that the seriousness of the morbid symptoms occurring in man are dependent on certain properties of influenza strains.

In general we assume that the seriousness of the syndrome is dependent on a combination of at least three factors:

a. the physical condition of the patient.
b. the number of "organisms" with which he is infected.
c. the property usally referred to as "virulence" (see below).

In the same way one may resolve the seriousness of an epidemic into a number of corresponding factors:

a. the state of health of the community in which the epidemic occurs;
b. the relative collective immunity;
c. the virulence of the infecting agent.

Usually we are too late to examine the state of health of the patient or community exhibiting the disease. Moreover, it is difficult to define in retrospect the number of germs with which the patient was infected. Hence our research made no attempt to go into these points, but was concentrated on specific collective immunity. In the first place we investigated whether there could have been any reason why an infection among the group studied should be transmitted more rapidly and more intensively than elsewhere. We were unable to envisage convincing arguments for it. Contact between patients had not been intensive. The capacity of the rooms in which they had been by day as well as by night was sufficient. The mental condition of the patients may have increased the chances of transmitting the infection, but this was negligable in the case in point.

It goes without saying that it was important to know the immunity condition of the group when the epidemic began. We were able to obtain some idea of this by studying the serological material (see above). In general, when one succeeds in isolating infectious agents, one tries to establish whether peculiar properties of the strain concerned can be brought into relationship with the course of the disease in the person from whom the virus was obtained. Comparison of various strains of an infectious agent in this way, may be defined as "determination of virulence".

The term occurs in bacteriology as well, and is the subject of much confusion. Many authors, for instance, use the words "pathogenic" and "virulent" synonymically (32). Before going on to discuss the subject in detail, it would be as well for me to air my personal views on the matter briefly.

I visualise virulence as a property of a certain bacterial strain (or virus strain). Research into virulence comprises comparative examination of bacterial (or virus) strains. This may consist of investigating whether certain properties are found in the bacteria (qualitative examination). In addition it may sometimes be possible to determine quantitative factors (e.g. investigation into the amount of toxin produced by C. diphteriae). Analysing the notion of "virulence" further, we may say that two groups of properties must be present in the bacteria in order to establish it as a virulent strain:

In the first place the power of invading the victim and maintaining itself there — "invasiveness", and in the second place the power of stimulating a morbid condition — "pathogenic" (12).

Any examination into virulence should include the determination of the number of bacteria (or virus particles) involved in the experiment, as well as the two properties mentioned above.

I suggest incorporating these factors into a formula which might have the following form:

$$\text{Virulence} = \frac{I_1 \times I_2 \times \ldots\ldots}{M} \times (P_1 + P_2 \ldots\ldots)$$

where I_1, I_2 etc. are factors determining invasiveness, all of which must be present, since the absence of any one of them would render the bacteria harmless; M represents the minimum number of bacteria (virus particles) that is necessary to bring about an infection; P_1, P_2 etc. are the various properties giving rise to a morbid condition (e.g. in diphteria the faculty of forming toxin and the property of initiating necrosis). Together they represent "pathogenity". It is evident that all the pathogenic factors need not to be present at the same time.

Defining the virulence of a bacterial strain does not always

involve going into all the factors that affect "virulence". It will often be possible to prove that some of the factors are present to a sufficient degree and that the virulence is more or less proportional to one strongly variable factor. For instance, when examining the virulence of C. diphteriae strains, usually only the faculty of forming toxin is measured (17, 26). Here lies a source of danger as on the one hand strains are known which are non-virulent through loss of invasiveness (Park Williams 8), whilst on the other hand very virulent strains occur which form little or no toxin. Usually these exceptions are not taken into account.

From the above it appears that virulence is a quantitative notion. A strain may be anything from non-virulent to extremely virulent. The aim is to try to express the concept of virulence in degree.

Any investigation into virulence concerns a comparison between strains of infectious agents. It is therefore essential that the strains concerned are indeed comparable. An example of two bacteria not comparable in this sense of the word are C. diphteriae and Streptococci. In general angina caused by the first will have a more serious course than that caused by the second, but this is far from establishing that C. diphteriae are more virulent than Streptococci. Similarly, we are not entitled to consider a difference in virulence between S. typhi and S. paratyphi B. From investigations into morbidity, mortality, duration of the disease, complications etc., the conclusion may be drawn that infection with S. typhi in man usually follows a more serious course than that with S. paratyphi B. It is quite possible, however, that the comparison of a not very virulent typhus strain with a very virulent Schottmüller strain would lead to the opposite conclusion.

The same reasoning applies to virus strains, which implies that the strains to be examined may not diverge too much from each other.

While we realise that certain kinds of bacteria are harmless to man but may increase in or on the human body, it is more difficult to envisage this situation in the case of an ultravirus, since we know that the latter increases in the living cell, mostly at the expense of the survival of that cell. The general condition of the individual, it is true, need not or need hardly be influenced by it, but we must be conscious of the fact that a number of cells will always fall victim to the virus. Consequently every virus proved to develop in the cells of an organism might be termed pathogenic for that organism.

Nevertheless pathogenity may vary from strain to strain. In defining virulence it will therefore be necessary to study the factors of invasiveness as well as those of pathogenity. In the formula mentioned above I have shown that virulence is inversely proportional to

the minimum number of virus particles necessary to cause morbid deviations. As regards bacteria this is very simple: one merely counts them. Although attempts have been made to count virus particles (16), serious difficulties still exist. It cannot be emphasised enough that defining the minimum infectious dose (we usually define the 50% infectious dose — essentially the same) is no indication of the number of virus particles found in the virus suspension.

In the one case, a single particle will be sufficient to bring on infection, while in another, a hundred or more will be required. Despite all this we are able to study a number of factors that play a part in the virulence problem. Firstly we can measure the rate at which the virus particles increase (or are increased) in the organism. This rate, at all events in the initial stages, will be independent of the number of virus particles causing the infection. We are able to do so by making use of Hoyle's experimental set-up (22). In Chapter V the results of experiments carried out by a similar method are given.

In addition to this, it is of interest to know something about the periodicity of the growth process of the influenza virus. These data are also important when defining virulence. Perhaps research into the intracellular development of the influenza virus may enlighten us in future about other factors of importance for the virulence problem. In chapter V an acount is given of research with object in view. How far the results of it can be used in the long run for the determination of virulence cannot be judged at the moment.

CHAPTER II

Some aspects of the laboratory diagnosis of virus diseases

Koch has taught us that a number of conditions must be fulfilled before we may designate a certain micro-organism as the cause of a certain disease (39). Recently we have been inclined not to take this too seriously.

Koch stipulated that the cause of a disease should be found in all cases and especially at the diseased sites. It is known to us now that the pathogenic organism may produce toxins that can be effective at a distance and that it is possible that the organism concerned may have disappeared by the time the symptoms become apparent.

Koch's second postulate is still less tenable than the first. It asserts that it must be possible to maintain the organism in pure culture for several generations, outside the body of the original host (leprosy).

Finally, Koch required that the micro-organism should be capable of imitating the morbid symptoms of the natural host in a suitable laboratory animal. This requirement is hardly ever fulfilled.

In the course of times several of Koch's original postulates have been changed and a number have been added. Julius, for instance, mentions that the organism in question must not be present in individuals of the same species not suffering from the disease (25). This view also proved untenable, for example by the virus of the herpes simplex (labialis), and by the existence of germ-carriers. One of Julius' additions, that the serological reactions must tally, cannot be maintained in the long run. One may cite the absence of antibodies which the host might have formed had it been penetrated at another site, in diseases like bacillary dysentery, etc.

Although it is possible to find exceptions for all rules regarding diseases the cause of which we know with certainty, it is nevertheless unwise to reject the whole of Koch's teaching.

The diagnosis of a great many diseases caused by bacteria is much simplified by the application of modern methods (isolation of the "virus"). In many diseases the diagnosis can only be made in the laboratory (Salmonellosis). Epidemiological investigation has made grateful use of it, since our knowledge of the part played by germ-carriers in epidemiology is based on laboratory work.

For these reasons it is evident that the epidemiologist hopes to increase his knowledge by means of the laboratory study of the

diseases caused by filtrable viruses. Here, however, he meets with difficulties only partly solved as yet.

For most of the virus diseases, the methods of isolating the infectious agent are not reliable enough. This means that mere failure to isolate a virus has no diagnostic significance. It is known of a number of virales that they have ceased to be present in the organism by the time the disease reaches its peak. We have repeatedly been able to verify this point. Garglings from 43 patients, whom we could assume from the clinical symptoms and the results of serological examination to be suffering from influenza, were introduced into the amniotic cavity of incubated hen's eggs. In 24 cases inoculation took place within 24 hours of the appearance of morbid symptoms. In 22 out of these 24 isolation attempts we succeeded in obtaining the virus. In 17 cases the gargling was collected from 24 to 48 hours of the commencement of the disease. Here we were only successful in 6 cases. In addition we isolated another two strains from garglings taken at unknown elapsed times after the commencement of the disease. We booked no results from garglings collected more than 48 hours after the clinical symptoms had begun.

To these two drawbacks — unreliability of method and the short period during which the virus is excreted — a third may be added: the considerable expense of the method. The following table illustrates the large number of eggs required to isolate the influenza virus.

TABLE I

Thirty isolations of influenza virus from garglings

Number of isolations	Number of eggs positive	
	1st passage	2nd passage
1	6/6	
4	5/6	
7	4/6	
6	3/6	
5	2/6	
3	1/6	
2	0/6	5/5
1	0/6	4/5
1	0/6	2/5

The numbers of eggs only refer to the primary isolation. When, moreover, the properties of the isolated strains are to be investigated, still larger numbers of eggs are required. During the first passage only the amniotic fluid is at one's disposal. The harvest is usually slight and has a low titre. The quantity can only be increased when the virus has been adapted to the allantoic cavity.

We made a number of attempts to counter these difficulties by applying other methods for demonstrating the virus, which are described below. At the same time we hoped to remove another drawback. The isolation of the virus in eggs takes on the whole a good deal of time. As regards the influenza virus it usually takes 4 to 8 days (and sometimes even more) before we are in a position to record the results.

A. *The Complement Fixation Reaction for Demonstrating Antigen (C.F.R.A.)*

Techniques are in use for variola and foot-and-mouth disease which would mean a solution of the above problems if they could be applied to other viruses. Using a known serum containing antibodies to the virus suspected, a complement fixation reaction is executed with the contents of the suspect vesicles as unknown antigen. Although it is also possible to apply this reaction to the dried crusts of variola, the results are not so reliable. This is one of the methods we attempted to apply to the influenza virus. As antigen we had the garglings. The exact composition of the gargling fluid was of vital importance. We had to be sure it was not anticomplementary, and moreover that it could not exercise destructive influence upon the virus. To establish this we made a number of tests comprised in Table II.

TABLE II

Comparison of infection titres of a virus suspension stored under various conditions and diluted with various fluids (initial titre 100, 50% infection doses — I.D. 50)

Composition of fluid	Storage conditions	Residue Percentage I.D. 50
1. saline 0.9 %	24 hours 37°	25
2. saline 0.9 %	24 hours room-temp.	60
3. broth saline	24 hours 37°	75
4. broth saline	24 hours room-temp.	85
5. broth saline and saliva	used immediately	40
6. as 5	24 hours 4° C	0
7. as 5 with antibiotics	immediately	40
8. as 7	24 hours 37°	40
9. as 7	24 hours room-temp.	40
10. as 7	24 hours 4° C	40
11. saline, saliva and antibiotics	24 hours 4° C	less than 10

The 50% infection dose (I.D. 50) was calculated according to the method of Reed and Muench (31). To this end ten groups of 6

eggs were inoculated with 0.1 cc of successive threefold dilutions of the initial virus suspension, commencing with a dilution 10^{-6} and ending with one of 2×10^{-5}. In the tests mentioned in Table II, 100 I.D. 50 per cc was added to the fluid to be tested. After the tests another titration was done this time in five groups of 4 eggs which were inoculated with 0.1 cc of the fluid in the following dilutions: — 1:200, 1:65, 1:20, 1:6 and 1:2.

The virus suspension used consisted of a pool allantoic fluid from eggs infected with an influenza A strain (Karsten).

It became evident to be that after an initially rapid decline the infection titre remained practically constant for some six weeks (storage in separate tubes at 4° C). The infection titre of the virus suspension was checked each time before and after the tests.

From Table II it appears that low concentrations of influenza virus are quickly destroyed when suspended in saline. For instance, a suspension containing 100 I.D. 50 per cc falls to a quarter of the original titre within 24 hours (stored at 37° C). At a lower temperature (room-temperature, about 18° C) the process takes place somewhat less quickly and more than half of it remains after the same period. By adding a little colloidal matter destruction is delayed considerably.

In this case we mixed saline with equal parts of a broth-peptone-saline mixture as used as a culture fluid in bacteriological laboratories. Within 24 hours (stored at 37° C) the I.D. 50 fell off to three quarters of the original titre and at a lower temperature (18° C) even more remained (25%). When, however, saliva was added to the broth-peptone-saline mixture in a proportion of 1 to 10, and stored at a low temperature (4° C), no virus could be detected after 24 hours. We are of the opinion that that must be ascribed to the considerable growth of bacteria. However, the saliva in itself also exercises a destructive influence, since immediately after adding it the I.D. 50 falls from 100 to 40 (Table II, 5). By adding antibiotics the growth of bacteria may be checked almost completely. To this end we used penicillin (400 U per cc) and streptomycin (dehydro-streptomycin 10 mgr per cc). The I.D. 50 then remained constant for 24 hours independently of the temperature at which it was kept. Because of the fact that much organic material is present in gargling- and saliva we examined the behaviour of the virus in saline to which pharyngeal mucus and antibiotics had been added (Table II, 11). Finally we carried out tests with broth-saline to which antibiotics had been added.

At first the presence of broth gave rise to technical difficulties. The gargling fluid was unpleasant to taste and an inconvenient quantity of froth was developed during gargling. We removed this

drawback by using two fluids: a washing fluid consisting of saline to which antibiotics had been added (unpleasant enough in taste for even mentally difficult patients to avoid drinking it) and a broth-peptone mixture in the receptable. The mixture has no anti-complementary properties.

Before going on to the complement fixation reaction (C.F.R.A.) we had to consider the further difficulties with which we might yet be confronted. From tests with garglings of normal individuals it became evident that the mucus and saliva sometimes confer anti-complementary properties on the fluid. These properties are usually removed by centrifuging the garglings for half an hour at 3000 r.p.m.

When we examined a number of sera with non-complementary garglings from normal individuals we encountered a severe set-back. Complement fixation with ferret immune sera usually produced a positive result. Fortunately, however, no fixation occurred with rabbit hyperimmune sera. We drew the conclusion that in the ferret sera antibodies to certain components of the garglings from normal individuals were present, which were not present in our rabbit sera. They were probably of bacterial origin.

The titre of each serum with regard to a number of homologous influenza strains is previously determined in the C.F.R. In the C.F.R.A. we use a twelve-fold concentration.

The next step was the C.F.R.A. examination of garglings from normal individuals, to which influenza virus had been added. To eliminate as far as possible the influence of components that would not be present in the garglings of influenza patients (egg material) we purified the virus suspensions. This we did in two ways: firstly by precipitating with methanol, centrifuging and resuspending (9), and secondly by adsorption to chicken's erythrocytes and elution. The suspension prepared by adsorption and elution contained 7.5 times as much I.D. 50 per agglutination unit (the minimum virus concentration that agglutinates ¼% chicken's erythrocytes completely) than the preparation purified with methanol. On the other hand, when determining the minimum concentration of the virus with which fixation of the complement could be obtained, it appeared that correlation existed between the C.F.R. antigen unit and the agglutination unit. The suspension prepared by means of adsorption to chicken's erythrocytes contained more "living" virus per C.F.R. antigen unit than the other, which means that the C.F.R.A. may also be executed with antigen no longer suitable for the reproduction process.

In Table V the results of the C.F.R.A. with garglings of (influenza?) patients are summarized. As the number of garglings was on the low side we increased it with nasal washings from ferrets,

previously infected intranasally with influenza virus (Table VI and VII). In these tables the results of another experiment are inserted for reasons of comparison. A discussion of the results follows after the description of a second method investigated.

B. Specific-Antibody Fixation (S.A.F.)

From the beginning we were rather sceptical of the likelihood of finding a solution of the problem using the C.F.R.A.. and we had remained on the lookout for other methods. One of these, based on Castellani's absorption test, we selected for further investigation. It was named Specific-Antibody Fixation (S.A.F.).

It is known that in antigen-antibody reactions no constant ratio is found between these two components. On the one hand a considerable surplus of antigen is necessary to remove all antibodies from a serum, while on the other hand a small quantity of antigen may suffice to fix a very large quantity of antibodies. We tried to make use of the latter principle. Here again we bring together unknown antigen (influenza virus) with a specific antiserum. The fixation having taken place, we titrate the surplus antibodies in the serum by means of the haemagglutination-inhibition reaction. For the S.A.F. it is necessary that as many antibodies as possible are fixed by a small quantity of antigen. At least two factors are known which affect the antibody-antigen reaction. Firstly the temperature at which the reaction takes place, and secondly the period of time during which they may interact. Most reactions take place more rapidly and more completely at 37° C than at lower temperatures. For some bacterial reactions still higher temperatures are used (Salmonellas). For the C.F.R. with virus antigens a long incubation period is used. As it is usually not possible to maintain absolute bacterial sterility in serological reactions of this type the work cannot be done at a temperature of 37° C. Nor does the adding of antibiotics prevent the danger of bacterial pollution. For this reason the reaction is brought about in the refrigerator. We were given the choice between a temperature of 56° C or higher (at which hardly any bacterial growth can take place), incubating at 37° C, but in that case not longer than 2 hours, or working at lower temperatures for a long time.

We have examined the matter of optimum conditions in three sets of experiments. In the first group (see Table III) we caused a constant quantity of virus (8 agglutination units) to act upon increasing serum dilutions for various periods of time and at 5 temperatures. Erythrocytes were added subsequently to determine the ultimate dilution at which the serum had fixed the virus under

the circumstances given (column 2). The influence of temperature and time upon 8 agglutination units of virus were established separately (column 3). The virus titre fell to about a half after maintaining a temperature of 56° C for half an hour, and to less than one-eighth of the original titre after 16 hours. Upon maintaining 80° C for half an hour, the haemagglutinating power was lost completely. In one experiment (not inserted into the table) it was observed that the fixation power of the serum does not change even when kept for half an hour at 80° C. It follows from the experiments mentioned in Table III that a small quantity of serum is capable of fixing a relatively large quantity of virus under certain circumstances (column 2). The optimum fixing result was attained after a protracted reaction at low temperatures. The haemagglutination titre of the virus did not fall at all during the course of this process (column 3).

TABLE III

The influence of temperature and time upon the fixation between antigen and antibody

Conditions	Constant quantity of virus		Constant quantity of serum
	H.I. titre of the serum	Haemaggl. titre of the virus	Haemagglutination titre of the unfixed virus
½ hour at 80° C	×	0	—
½ hour at 56° C	2400	4	60
16 hours at 56° C	×	1	—
½ hour at 37° C	1200	8	120
2 hours at 37° C	2400	8	60
4 hours at room-temp.	1200	8	240
½ hour at room-temp.	600	8	480
4 hours at 4° C	600	8	120
16 hours at 4° C	4800	8	60

H.I. = haemagglutination inhibition
× = not determinable (exhibits no (complete) haemagglutination).

In the second series of tests we investigated the quantity of virus that may be fixed by a constant (small) quantity of serum. We added serum diluted 150 times to a series of virus dilutions (see Table III, column 4). A lower haemagglutination titre means that more virus is fixed by the serum, except in the first three experiments, where virus is destroyed (see column 3).

As in the previous experiments the influence of time is clearly perceptible. The longer the reaction lasts, the more virus is fixed. The temperature effect is stronger here than in the other series.

Two hours at 37° C gives the same result as 16 hours at 4° C (column 4). In this test series it was also investigated under which circumstances a given (fairly small) concentration of antibodies fixes the largest quantity of virus antigen. We know that in certain other serological reactions the fixation between serum and antigen becomes optimum under certain external circumstances, it being quite immaterial whether a large quantity of antigen and a small quantity of antibodies are concerned or vice versa (e.g. toxin-antitoxin titrations). But to be quite certain we investigated the latter situation separately in a third series of experiments (Table IV).

TABLE IV

The influence of time and temperature on the degree of fixation of specific antibodies by several concentrations of influenza virus (A′) in an anti-influenza A′ serum

Conditions	concentration of the virus (in agglutination units) added to the serum before the experiment						
	0	⅛	¼	½	1	2	4
1. ½ hour at 56° C	3840	3840	3840	3840	3840	2880	1440
2. 2 hours at 45° C	3840	3840	3840	2880	1920	1440	960
3. 2 hours at 37° C	3840	3840	2880	1920	1920	1440	960
4. 2 hours at 18° C	3840	3840	3840	3840	2880	1920	1440
5. 2 hours at 4° C	3840	3840	3840	3840	3840	2880	1920
6. 16 hours at 4° C	3840	3840	2880	1920	1440	1440	960
7. combination of 2 and 6	3840	3840	2880	1920	1440	1440	960
8. combination of 3 and 6	3840	2880	1920	1920	1440	960	960

The object of this series was to find out under which circumstances a small dose of virus fixes the largest quantity of antibodies. To this end, increasing concentrations of influenza A′ virus were added to anti-influenza A′ serum, and the mixtures of serum and virus treated under various conditions of time and temperature (see column 1). After "saturation" of the serum we conducted a haemagglutination inhibition test to determine the residual quantity of antibodies. A lower titre indicates that a greater proportion of the antibodies have been fixed. Here again the influence of temperature and time is obvious. The combination of a short period at 37° C and a long period at 4° C gave the best result (series 8). From these results we may conclude that a small quantity of virus will be capable of fixing a relatively larger quantity of antibodies, provided the conditions are favourable. What may upset the results badly is the chance of bacterial growth, as the material (gargling) must be deemed to be seriously infected with bacteria. This was countered by causing the action of the antigen upon the serum to take place for the greater part of the time at a low temperature.

Possessed of these data we were now in the position to carry into effect the principle of Specific-Antibody Fixation (S.A.F.). The object is to cause material containing virus (artificial gargling to which virus is added) to act upon an anti-influenza serum, after which the titre of the residual antibodies is determined by a haemagglutination inhibition test with a known dose of virus. The practical set-up is the following: A series (1, 2, 4, 8, ...) of dilutions of a known anti-influenza serum was made using virus-containing material (mixture of broth, saline and antibiotics to which purified virus and saliva is added) as dilution fluid. As a check a series of dilutions of the same serum was made with the mixture of saline and antibiotics without additives. In some cases we set up an additional check in which the artificial garglings were used, without adding virus to the diluting fluid. We discontinued this latter titration check after it became evident that when it deviated from other titration checks, it always showed a higher haemagglutination inhibition titre. We used anti-influenza A and B serum and always duplicated the titrations. In the anti-A serum titration the residual antibodies were, of course, titrated with A virus and the anti-B serum with an influenza B strain. The S.A.F. reactions which were executed with the non-appropriate serum and virus, could moreover serve as a check. We experimented further along the same lines to arrive at the best possible set-up. This also required that the influence of temperature and time was investigated. Finally it appeared best to proceed as follows. First we incubated the series of dilutions in artificial garglings with virus at 37° C for two hours. Next the racks were stored in the refrigerator during the night and then placed in a water bath at room temperature for half an hour. The erythrocyte suspension and the 4 haemagglutination doses of the virus appropriate to the serum were then added successively. The result could be read after the tubes had been left for half an hour or an hour at room temperature. In the test series executed with A′ virus, we encountered some initial trouble through the property of this special virus of easily being fixed by non-specific antibodies. However we were able to reduce this difficulty by making the virus and erythrocyte suspensions in a 1% citrate 0.6% sodium chloride solution, adding the blood cells first, followed by the virus. The sensitivity of the reaction was further improved by subjecting the artificial garglings to a similar treatment as described above for the C.F.R.A. heating for half an hour at 37° C in a water bath and then centrifuging at 3000 r.p.m.. The serum dilutions were then made with the supernatant fluid.

The next stage was to establish the sensitivity of the set-up. It appeared to be much greater than that of the C.F.R.A.. The latter

required at least 100 agglutination units, while the S.A.F. already provided a positive result with ¼ agglutination dose per cc added to the artificial garglings. As in the case of the C.F.R.A. there was no difference between the two virus suspensions prepared and purified by different methods. Hence it is unnecessary for the S.A.F. as well that the virus present in the garglings should possess reproductive qualities.

In a number of check tests we attempted to ascertain the reliability of the method. To this end we executed 100 titrations with the same artificial garglings. In 6 cases a twofold divergence from the average was found (only higher values). In 10 cases a measurable divergence was found, but less than twofold (7 higher, 3 lower). In 84 out of the 100 cases the same titre was read. As this is not a typical Gaussian distribution, it is not possible to predict how often a fourfold deviation would occur. In addition it is difficult in practice to read partial agglutinations. Consequently the intermediary values are irregularly distributed and we may not rely upon their accuracy.

Since it is necessary to set a borderline between positive and negative, we nevertheless accepted the twofold divergence as minimum for two reasons. Firstly because we duplicated the titrations and secondly because we were able to use the titrations of the heterologous serum as a check. In the course of the investigation it appeared that with a positive S.A.F. (= a twofold or greater decrease in titre of the homologous serum) the titre of the heterologous serum was often increased.

In Table V we have summarised the results of the work done with the S.A.F. and the C.F.R.A. on garglings of (influenza) patients. To our regret we were not able to perform all the reactions simultaneously as we often ran out of gargling fluid. Shortage of eggs of the required age also prevented us at times from carrying out isolation attempts.

In columns II to X the results of the various methods of investigation are stated successively. Column II contains the results of the examination of the acute phase sera and immune sera collected from the patients. A fourfold or greater rise in the titre of the second serum compared to the first serum was assessed to be a positive result. By each positive result, the virus group for which it was positive is entered in parentheses. The garglings were used to inoculate eggs. Where an influenza virus was isolated it is entered in column III stating the type of the strain. The corresponding C.F.R.A. reactions are entered in columns IV and V. Columns VI to X refer to the S.A.F. results. In columns VI and VII the results of the duplicate S.A.F. with anti-A serum are set out, and those for anti-B serum in columns VIII and IX. The figures represent the ratio of

the titre to the titration check of the sera used. For instance, where the titre with the gargling as diluting fluid was 480, and the titration check was 1920, ¼ has been entered as the result. The conclusions drawn from the S.A.F. results are inserted in column X.

TABLE V

I	II	III	IV	V	VI	VII	VIII	IX	X
			C.F.R.A.		Specific antibody Fixation. Ratio of titre to check titration				
Patient	serolog. examin.	isolation	anti infl. A serum	anti infl. B serum	anti A serum		anti B serum		conclusion
1	?	+A′	—	n.c.	1/4	1/4	1	2	+(A)
2	+(A)	+A′	+	n.c.	1/8	1/6	1	1	+(A)
3	+(A)	—	+	n.c.	1	1	1	1	—
4	+(A)	+A′	—	n.c.	1/4	1/4	2	2	+(A)
5	+(A)	+A′	—	n.c.	1/4	1/4	1	1	+(A)
6	?	—	+	n.c.	1	1	1	1	—
7	+(A)	—	—	n.c.	1	1	1	1	—
8	+(A)	—	+	n.c.	1	1	2	2	—
9	+(A)	+A′	+	n.c.	1/4	1/4	1	1	+(A)
10	+(A)	—	+	n.c.	2	2	2	2	—
11	—	—	+	n.c.	1	1	1	1	—
12	± (2fold A)	+A′	—	n.c.	2	2	2	2	—
13	+(A)	—	+	n.c.	1	1	1	1	—
14	?	—	+	n.c.	1	1	1	1	—
15	+(A)	+A′	—	n.c.	1/2	n.c.	1	n.c.	± (i.m.)
16	—	+A′	—	n.c.	1/2	n.c.	1	n.c.	± (i.m.)
17	+(A)	+A′	+	n.c.	1/2	1/2	1	1	+(A)
18	—	—	+	n.c.	1	1	1	1	—
19	?	—	+	n.c.	1	1	1	1	—
20	—	—	+	n.c.	1	1	1	1	—
21	—	—	+	n.c.	1	1	1	1	—
22	?	—	+	n.c.	1	1	1	1	—
23	+(A)	—	+	n.c.	1/2	1/2	1	1	+(A)
24	—	—	+	n.c.	1	1/2	1	1	—
25	+(A)	+A′	—	n.c.	1/2	1	1	1	— (i.m.)
26	+(A)	+A′	+	n.c.	1/2	1/4	1	1	+(A)
27	+(A)	b.p.	—	n.c.	1/2	1/6	1	1	+(A)
28	+(A)	b.p.	o.f.	n.c.	1/2	1/6	1	1	+(A)
29	+(A)	+A′	+	n.c.	1/2	2/3	1	1	—
30	+(A)	n.c.	—	n.c.	1	2	1	1	—
31	—	n.c.	+	n.c.	1	1	1	1	—
32	—	n.c.	+	n.c.	1	1	1	1	—
33	—	n.c.	+	n.c.	1	1	1	1	—
34	+(A)	—	—	n.c.	1/3	1/3	1	1	+(A)
35	—	n.c.	+	n.c.	1	1	1	1	—
36	+(B)	—	—	—	1	1	1/3	1/4	+(B)
37	+(B)	+B	—	+	1	2/3	1/8	1/8	+(B)
38	+(B)	—	—	—	1	1	1/4	1/4	+(B)
39	+(B)	—	+	+	1	1	1/3	1/4	+(B)
40	+(B)	+B	—	+	1	1	1/2	1/2	+(B)
41	+(B)	+B	—	+	1	1	1	1	—

n.c. = not carried out o.f. = own fixation b.p. = bacterially polluted.
i.m. = insufficient material ? = unknown

In Table VI we have tried to present the results in a somewhat clearer manner.

TABLE VI

Comparison between various diagnostic methods using garglings.

		CFRA + SAF +	CFRA + SAF —	SAF + CFRA—	both reactions —
isolation positive	15	5	3	3	4
isolation — but serol. examination +	13	1	4	6	2
isolation — and serol. examination —	13	0	13	0	0

Applying these methods to 41 garglings we succeeded in isolating the virus 15 times. Four of them gave negative results with both the C.F.R.A. and the S.A.F. In 5 cases both reactions were positive. In 6 cases (3 and 3) one of the two *in vitro* methods turned out negatively. On the other hand, titration gave a positive result in 11 cases, where the egg inoculation failed. In thirteen garglings from individuals in all probability not suffering from influenza, complement-fixing antigen was demonstrated. We are of the opinion that these latter have to be considered non-specific.

We had difficulty in obtaining sufficient material for the investigation. Accordingly we included nasal washings from infected ferrets. The washing fluid was the same as previously used.

The anaesthesized animals were held with their noses over Petri dishes. A. Pasteur's pipette bent slightly at the end and the sharp edge rounded off in a flame, can easily be introduced into the nasal cavity. The pipette is held vertically. The contents of it now flow into the nasal cavity under slight pressure, wash the mucous membranes and are collected in the Petri dish. The amount of fluid used per washing was 12 cc.

The results of these examinations have been summarized in Tables VII, VIII and IX. In Table VII, I have tried to give an impression of how the work was carried out by quoting a number of typical series. Column III shows the result of comparative serological examination of the sera gained respectively before and a fortnight after the infection. The contents of the other columns are the same as those in Table VI.

TABLE VII

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
ferret no.	virus with which infected	result of comp. ser. gained	date of collection of material	result of virus isol.	C.F.R.A.		Specific Antibody Fixation				conslusion	
					A	B	A		B		A	B
31	A	+	D	—	—	—	1	1	1	1	—	—
			D+1	+	—	—	1	1	1	1	—	—
			D+2	+	—	—	1/2	1/2	1	1	+	—
			D+3	+	+	—	1/4	1/4	1	2	+	—
32	A	+	D	—	—	—	1	2/3	1	1	—	—
			D+1	+	—	—	1	1	1	1	—	—
			D+2	+	+	—	1/2	1	1	1	—	—
			D+3	+	+	—	1/8	1/8	1	2	+	—
33	A	+	D	—	—	—	1	1	1	2/3	—	—
			D+1	+	+	+	1	1	1	1 1/2	—	—
			D+2	+	+	+	1/4	1/4	1	2	+	—
			D+3	—	+	+	1	2	2	2	—	—
34	A	—	D	—	—	—	1	1	1	1	—	—
			D+1	—	—	—	1	2/3	1	1	—	—
			D+2	—	—	—	1	1	1 1/2	1	—	—
			D+3	—	—	—	1	1	1 1/2	1	—	—
35	B	+	D	—	—	—	1	1	1	1	—	—
			D+1	+	—	+	1	1 1/2	1/4	1/8	—	+
			D+2	+	—	—	2	2	1	1	—	?
			D+3	—	—	—	2	2	2	1 1/2	—	—
36	B	+	D	—	—	—	1	1 1/2	1	1	—	—
			D+1	+	—	+	1	1 1/2	1	1 1/2	—	—
			D+2	+	+	+	2	2	2	1	—	—
			D+3	—	+	+	2	2	2	1 1/2	—	—
37	B	+	D	—	—	—	1	1	1	1	—	—
			D+1	+	—	—	1	1	1/2	1/2	—	+
			D+2	+	—	—	1	1 1/2	1 1/2	1/2	—	+
			D+3	—	—	—	2	2	1 1/2	2	—	—
38	B	+	D	—	—	—	1	1	1	1	—	—
			D+1	+	—	+	1	1	1/2	1/3	—	+
			D+2	+	+	+	1 1/2	1 1/2	1/2	1/2	—	+
			D+3	+	+	—	2	2	1 1/2	1 1/2	—	—
17	none	—	D	—	—	—	1	1	1	1	—	—
			D+1	—	—	—	1	1	1	1	—	—
			D+2	—	—	—	1	1	1	1	—	—
			D+3	—	—	—	1	1 1/2	1	1 1/2	—	—
			D+4	—	+	+	1	1 1/2	1 1/2	1 1/2	—	—
			D+5	—	—	+	1 1/2	2	1 1/2	1 1/2	—	—
			D+6	—	+	+	1	1 1/2	1	2	—	—

D = date of infection.

As regards the S.A.F. results it is noticeable that up to D + 1 the values obtained from the washings and heterologous serum tally

well with the titres of the check determination with that serum. Later values, however, from washings and the non-appropriate serum tend to rise above those of the check test. From this the conclusion could be drawn that the nasal washing comes to contain non-specific haemagglutination inhibitive material. This was also the case with uninfected ferrets, of which no. 17 (Table VII) is an example, the rise in titre appearing at D + 3. As it was proved by a few tests that repeated anaesthesizing had no influence upon the nasal mucus we may assume that repeated washing of the nasal cavity is the cause, probably giving rise to an inflammation. Usually after a few days we began to observe more mucus and sometimes even a little pus. This also shows in the C.F.R.A. results, which become falsely positive. This cannot be caused by non-specific complement-fixing material, as these would have been observed in the check-test without serum. In our view the cause lies in the presence of bacterial matter.

TABLE VIII

Comparison between various methods by which influenza virus (antigen) can be demonstrated. Material: nasal washing from ferrets

date of examination	number of specimens examined	virus strain	positive serol. results	isolation positive	C.F.R.A. positive	S.A.F. positive
D+1	16	A		12	2	4
D+2	16	A		13	10	8
D+3	16	A		12	6	9
D+4	16	A		4	1	0 2x?
D+5	8	A		1	0	0
D+6	8	A		0	1	0
Totals:	16	A	14	15	12	13
D+1	12	B		10	7	6
D+2	12	B		10	5	7
D+3	12	B		6	3	4
D+4	12	B		2	2	0 1x?
D+5	12	B		0	3	0
D+6	12	B		0	1	0
Totals:	12	B	10	11	12	8

D = date of infection.

In Table VIII the various results are compared. In 3 cases we obtained a doubtful result with the S.A.F. We found that the haemagglutination inhibition test executed with the heterologous serum and the nasal washing showed a more than twofold increase in titre

compared with the check titration of the same serum, while the titre of the homologous serum exhibited no change. Hence it is possible that a specific titre decrease of the homologous serum is masked by non-specific haemagglutination inhibiters.

These three doubtful results have no influence on the totals, as specimens from the ferrets concerned had already been positive the previous day.

Finally the false reactions have been summarized in Table IX.

TABLE IX

False results from ferret nasal washings

	virus strain	number of examinations	C.F.R.A.	S.A.F.
1. False positives				
	A	216	33	1
	B	224	43	2
Totals:	A or B	440	76	3
2. False negatives (virus isolation positive)				
	A	42	19	23 (21)
	B	28	15	12 (11)
Totals:	A or B	70	34	35 (32)

Here it appears that, contrary to expectation, the S.A.F. shows no difference in sensitiveness to the C.F.R.A., though there was a clear difference in the number of false positives. Out of 440 examinations 76 were returned as false positives by the C.F.R.A. method. A percentage as high as this (over 17%) renders "positive" inconclusive. The S.A.F. on the other hand, exhibited only 3 positives in 440 examinations (1%) that could not be brought into line with the remaining results. It was to be expected that the reaction would not be as sensitive as isolating the virus by inoculating. It is difficult to establish what percentage of inoculations fail. We occasionally succeeded, however, in transmitting the virus from one ferret to another 6 days after infection at a time when eggs inoculated with ferret nasal washings had already ceased to yield results. From this it appears that positive results are also dependent on the infectiousness of the virus for the test animal examined.

Another problem occurred when we began to use another virus strain. Our earlier work was done with a strain isolated by us personally (Khaut I), and belonged to the "Liverpool" type (see Chapter IV). When, sometime later, we changed over to another strain of the

"Stockholm" type, a good many S.A.F. reactions began to turn out negatively. We are unaware whether this was due to the antiserum used or to the peculiar properties of this strain.

In addition to those described above we carried out a number of other *in vitro* experiments for detecting influenza virus. In the first place we tried to concentrate the virus from garglings in various ways. Adsorption to and elution from chicken and human erythrocytes proved to be unsatisfactory.

The Cox methanol-precipitation method (9) of concentrating virus was applied to allantoic fluid previously added to garglings. The suspension yielded was capable of agglutinating erythrocytes, which process was inhibited by the appropriate serum. But tests with material naturally containing virus failed (no agglutination). This cannot have been due to insufficient virus as there was clearly enough to infect eggs even when diluted to 10^{-6}.

We also investigated whether erythrocytes added to the garglings under various conditions underwent any specific changes. We discovered that they became panagglutinable in a number of cases, that is to say were agglutinated by all the sera examined, but it was not possible to develop a specific reaction with them.

We had hoped to be able to set up an investigation into "virus carriers" by means of one of the methods discussed above. We intended to make use of our method as a screening test in the same manner as the Kline test is used in the examination of lues. From what has been said it will be apparent that none of the techniques described are suitable for this purpose. In the first place they are not sensitive enough, and in the second place they sometimes give negative results even when sufficient virus is present. Finally the only method that might be considered applicable, the S.A.F., is complicated and laborious.

In conclusion, we may say that the S.A.F. may in some cases be considered a valuable expedient for demonstrating virus. For instance, it can be applied when egg inoculation is ruled out, or when a rapid diagnosis is required (S.A.F. gives results within 24 hours). Moreover it might be possible to apply the principle to other virales.

Finally we have attempted to evolve a micromethod for demonstrating antibodies in blood. The great drawback of the usual method is that blood must always be obtained by venapuncture. Often at least 5 or 10 cc of blood are drawn off, when only a few drops of serum are required. Many people consider a venapuncture a drastic and unpleasant procedure, and when repeated serological reactions are required, this generally proves an insuperable obstacle. Sir

Almroth Wright's solution to the problem involved a piece of glass tubing with a gooseneck blown on to one end (41) and does in fact enable one to gain the necessary serum from a few drops of blood from the ear lobe or the fingertip. In the long run, the method described by him is disappointing, since making the pipettes requires great accuracy and takes time. They are very fragile and can only be used once. Moreover we found that the method requires considerable experience and even when that is available, it does not give always a good result. Hence our efforts had to be devoted to the search for an alternative method. We tried to make use of the property of the influenza virus when adapted to eggs of agglutinating human erythrocytes. As with chicken erythrocytes, the agglutination of human red blood cells by influenza virus is specifically inhibited by appropriate antibodies. It is know that with this reaction the titres, contrary to many other serological reactions, are not only dependent on the concentration of the antibodies in the serum, but within certain bounds are inversely proportional to the concentration of the virus as well. To elucidate this, we give below a graph in which the determination of inhibition is plotted as a function of the concentration of the virus and the dilution of the serum (Fig. I). Athough it is clear that twice as much serum fixes more than twice as much virus, it is equally clear that a constant relationship between these two components exists.

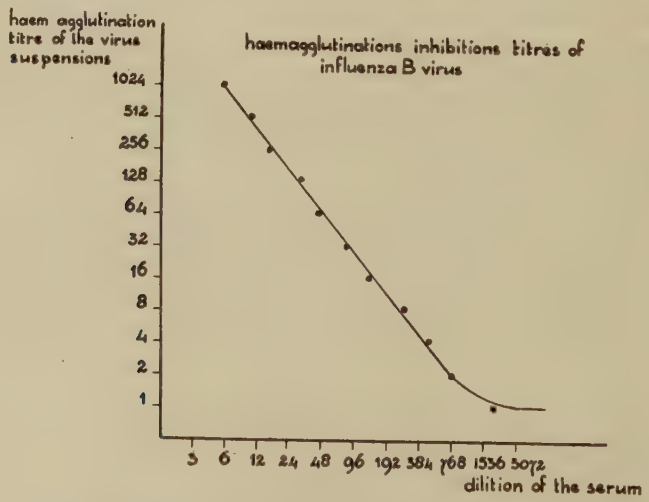


Fig. 1

If we take the above into account it must be possible to develop an agglutination inhibition reaction with the serum of the patient and his own red blood cells, working with a constant dilution of

the blood and various concentrations of virus. Of course it is necessary to render the blood non-coagulable, but in that case we are concerned with plasma and no longer with serum. Hence it was necessary to investigate whether the agglutination inhibition test could be executed with plasma instead of serum. No difficulties were encountered here. This led us to collect the blood of the patient in a citrate solution and to dilute it in such a way that the final concentration of the red blood cells in a series of test tubes is ¼%, and to add to each an increasing quantity of virus. The method enables the obtaining of an impression of the quantity of influenza antibodies circulating in the patient's blood. Though it can be used as an informative reaction, one must not expect the values obtained by it to compete with the results obtained by the A.I.T. or by the C.F.R. It is an approximate estimation. To our regret we were only able to apply this test to some dozens of blood samples and compare them with the results of other methods. We hope to collect sufficient material during a future influenza epidemic to check reliability.

Not being sure of the efficacy of the above procedure we developed yet another micromethod. A normal C.F.R. can be carried out with citrate plasma of blood obtained from the finger. If desired a micro-C.F.R. may be utilized (11, 18). Compared with the first method however, this is elaborate and therefore less suitable for the purpose in question (43).

APPENDIX

The techniques for the procedures discussed in Chapter II for demonstrating virus and for the serological micro-reactions.

A. Egg inoculation.

The primary isolation of influenza virus using the incubated hen's egg takes place by means of amnotic inoculation. The eggs are incubated in a normal incubator for 9 days in the case of influenza B virus, and 12 in the case of influenza A strains. It is not necessary to turn the eggs regularly, and a temperature of 38.8° C (101.8° F) is best for the purpose. They are then candled against a strong light source. The air chamber is marked, and in addition the eye of the embryo is sought out and a spot marked on the shell adjacent to the eye at a site where there are no blood vessels. These two marked sites are pencilled with a 5% tincture of iodine and holes are bored through the shell at both places with a dentist's drill. The drilled eggs are then kept in the incubator until they are inoculated. This we carry out with a hypodermic syringe and a long (serum) needle. The egg is held in front of the candling light and the needle inserted in the direction of the eye of the embryo until the latter is seen to be pushed aside. At this stage the point of the needle is partly in the embryo, and the opening partly in the amniotic cavity. The fluid being injected chooses the route of least resistance and flows into the amniotic cavity. This can easily be checked by adding a little methylene blue to the fluid to be injected, though we restrict this to demonstrations or as an expedient in teaching the technique. By this method one introduces from 0.1 cc up to as much as 0.4 cc into the amniotic cavity. After inoculation the openings are closed with molten paraffin wax (melting-point 50° C). In this simple way a considerable number of inoculations may be executed in a relatively short time.

The eggs are finally indexed in pencil on the shell and placed in another incubator this time at a temperature of 35° C (95° F). Again it is not necessary to turn the eggs regularly. We usually place them on indented papier maché egg-crating cards, so that the longitudinal axis of the egg is horizontal and the inoculation site uppermost.

Four days after the inoculation the eggs are refrigerated for two hours at 4° C, and the virus harvested. To this end the shell above the air-chamber is broken off with a pair of tweezers. First the allantoic fluid is removed with a Pasteur's pipette, to which a length

of tubing is attached for oral suction. A drop of the fluid is put into a tube containing glucose broth for checking bacterial sterility. Four drops are put into an agglutination tube, to which 1 cc ¼% of guinea-pig or human erythrocytes are added for the agglutination check. The rest is collected in sterile test-tubes. The upper part of the chorion allantoic membrane and the egg membrane are withdrawn by means of a fine pair of tweezers enabling a good view to be obtained of the embryo surrounded by the amniotic membrane. With a fresh Pasteur's pipette the contents of the amniotic cavity are drawn out and the same checks made as for the allantoic fluid. In some cases there is very little amniotic fluid and then we wash out the cavity with glucose broth and use the fluid obtained for further examination.

If the amniotic fluid in the agglutination tube causes the erythrocytes to agglutinate and there is sufficient fluid, a little is mixed with specific antisera with a view to checking whether the agglutination is inhibited thereby. The rest is used for further passages. If the haemagglutination test returns a negative result, the amniotic fluids of the various eggs are added together and used to carry out new amniotic inoculations. We always save a little of the yield from each tube in order to be able to repeat this "blind" passage if one of the fluids is bacterially unsterile. Moreover we always try to save some of the positive material for freeze drying.

B. The Complement Fixation Reaction for detecting Antigen (C.F.R.A.).

Complement. Freshly drawn or frozen guinea-pig serum (tubes filled with 1 cc serum and placed in the deep freezer at —30° C immediately after collection).

Sheep's erythrocytes. Defibrined sheep's blood, drawn off not longer than one week beforehand, is centrifuged shortly before use and washed. The viscid mass is considered to consist for 100% of erythrocytes.

Amboceptor. The titre is regularly checked.

Haemolytic system. The composition of the haemolytic system contains sheep's erythrocytes to a concentration of 1% and 10 units of amboceptor.

The serum. For our investigation we used rabbit sera. The rabbits had been subcutaneously injected with 10 cc of purified antigen, three times at 4 day intervals. The antigen was centrifuged at 9000

revolutions (about 12000 g) precipitated with 20% methanol and resuspended (haemagglutination titre at least 9000). Between ten to fourteen days after the treatment, blood was drawn off and checked for antibodies. Before the injection with antigen it was first established that the serum of the rabbit concerned did not contain any non-specific anti-bodies.

The serum was diluted such that at least 12 times the final concentration of the titre established in a check C.F.R. was present.

We were best satisfied by two sera prepared with a strain of the PR 8 group isolated in 1947 (H. W. from a laboratory infection).

Antigen. This was partly human (garglings) and partly ferret (nasal washings). The throat or nose was washed with fluid of the following composition: saline 0.9% in which 400 units of penicillin and 0.25 mgr of streptomycin per cc were suspended. Immediately after the washing operation, the fluid obtained was mixed with the same volume of bacteriological broth (peptone-saline-meat extract). First the antigen is heated at 37° C in a water bath for half an hour and then centrifuged half an hour in an angle centrifuge (Wifug) at 3000 r.p.m.

Dilution fluid. For diluting the antigen a fluid of the same composition as the washing fluid was used; a mixture of broth, saline and antibiotics. All the other dilutions are made with 0.9% saline.

Serum, antigen, complement and haemolytic system are used in equal quantities. Two preliminary tests are carried out: one with serum and the dilution fluid for the antigen, and one without. For the titration of the complement (preliminary test) a series of twelve dilutions are made containing respectively, 2%, 1.5%, 1.25%, 1%, 0.75% .... of guinea pig serum to twelve terms. The results of the two tests must not diverge by more than one dilution term. In the main test we use twice the exact unit complement as determined in the test with serum and dilution fluid. A series of four twofold dilutions of serum is made and the antigen, complement and a serum added. An antigen check without serum is also included, and the entire series put into the refrigerator at 4° C for 16 hours, as is the haemolytic system. Both are then taken out and put into a water bath at 37° C for half an hour. The haemolytic system is then mixed in, and the racks kept in the water bath for at least half an hour. The further reactions can now be read off. The first tube exhibiting complete haemolysis is taken as the limit for the reading.

The last tube exhibiting turbidity counts as the C.F.R. unit if the quantity of antigen is to be determined.

C. *The Specific-Antibody Fixation (S.A.F.).*

Dilution fluid.

When a dilution fluid is required, it is composed as follows:—
Sodium citrate 10 grammes
Sodium chloride 6 grammes
aqua ad 1000 cc.

Suspension of erythrocytes.

The reactions are executed with human O erythrocytes.

A ¾% suspension is used, the terminal concentration being ¼%. We usually obtain the human O blood from the blood bank, specifying that it should have been collected in half its volume of acid-glucose-citrate. This mixture keeps for a long time if stored in a refrigerator. For each test a fresh suspension is made by washing twice with citrate saline.

Antigen.

As described above for the C.F.A. test.

Check antigen.

The washing fluid, bacteriological broth with saline and antibiotics.

Virus suspension.

Purified allantoic fluid. It is sufficient to centrifuge the fluid for half an hour at 3000 r.p.m.

Sera.

Contrary to experience with the C.F.R.A., potent ferret sera appeared to be more useful here than rabbit sera. We had available sera against A′ and B (Lee) strains. The serum titres varied from 2000 to over 4000.

Execution: For each gargling or washing (antigen) 2 racks of each 10 tubes are used, one for A serum and one for B serum. In addition, each set of tests comprises two further racks of tubes using the check antigen as diluting fluid. Into each tube, ¼ cc of antigen is poured. A series of twofold serum dilution are made up, commencing with ¼ cc of 1 in 5. Half the contents of the ninth tube is thrown away, the tenth being used as a check. The racks are placed in a water bath at 37° C for two hours and then placed in the refrigerator for 16 to 20 hours. Subsequently they are placed in a water bath at about 20° C (room-temperature) for half an hour. In the meantime a titre determination for the (purified) virus suspension is carried out. A series of ¼ cc twofold dilutions is

made using broth-saline as a diluting fluid. To each tube ¼ cc of citrate saline is added and a similar quantity of erythrocyte suspension. Usually the titre can be read off as early as half an hour later. The concentration of virus in the last tube in which complete agglutination takes place is taken to be the agglutination unit. The virus suspension is then diluted such that each tube receives 4 agglutination units. Now we continue with the reaction proper. To each tube ¼ cc erythrocyte suspension and ¼ cc virus dilution are added successively and well mixed. The result can usually be read off after half an hour. Complete inhibition of the agglutination is taken as the terminal point. At the same time a check haemagglutination-dilution series is carried out on the virus suspension. The calculation of the agglutination titre is some times productive of error. Care should be taken to ensure that A′ virus is added to anti A′ serum and B virus to B serum.

If the titre of one of the sera in the test is clearly lower than that with check antigen it is a strong indication of the presence of virus in the gargling or washing under investigation. At the same time it is possible to read directly whether the infection is caused by A′ or B virus. The result is negative when the titres are equal or when they are lower with both sera. The latter case has not been observed up till now. In a number of cases the titre was lower with one serum and higher with the other. This is strong evidence that the patient concerned is undergoing infection by a strain of the group appropriate to the serum for which the titre is lower.

D. Micro haemagglutination-inhibition test.

Sodium citrate solution:	Sodium citrate	3.8 grammes
	Aq. dest.	100 cc

pH with citric acid to be brought up to 7.0

First draw 0.4 cc citrate into 1 cc pipette, such as is used for blood-sugar determination then draw up enough blood to reach the 1 cc line. The blood is obtained in the usual manner from the tip of the finger or the ear-lobe. The contents should then immediately be blown into a tube, and these mixed by drawing up again into the pipette and blowing it out again, without allowing air bubbles to form. The tube is closed by a rubber stopper.

As antigen in this reaction, influenza A and B virus suspensions are employed. The reaction can also be used for the mumps virus. The titre of the virus suspension is determined with human O erythrocytes. The virus-containing allantoic fluids are centrifuged for half an hour at 3000 r.p.m. and twofold dilutions are made in citrate

diluting fluid (see above). To this an equal volume of human O erythrocytes is added. After remaining half an hour at room-temperature the reaction may be read off. The dilution factor of the virus in the last tube exhibiting complete agglutination should be taken as titre. For any titre of the virus suspension, provided it is at least 1500, the micro-test is executed with allantoic fluid diluted twice, 8 times, 32 times, and 128 times. Hence, four tubes per antigen are used. In each of these tubes ½ cc of virus dilution is mixed with the same quantity of the citrate blood, which has been diluted forty times with a 1% citrate solution. The racks are then kept at room-temperature for half an hour, after which the reaction can usually be read off. If this is not the case the racks are placed into the refrigerator and read half an hour later. With blood from normal individuals agglutination usually occurs in all four tubes, though sometimes agglutination in the fourth tube is inhibited. When antibodies are present we find that agglutination is inhibited in more of the tubes.

It is not possible to express the result in a number, as the determination of the virus titre does not take place with the same red blood-cells as the test itself. We therefore indicate the result by a number of plus signs. The reaction is negative when agglutination has occurred in all the tubes. For each tube in which agglutination is inhibited, a plus sign is accorded (—; +; ++; +++; ++++).

E. The micro complement-fixation reaction (carried out with plasma).

Blood is obtained in the same manner as under D for the micro agglutination inhibition reaction. The tubes are left to stand for some time, or, if there is haste, they are centrifuged. The supernatant fluid is used for the ordinary C.F.R., but as plasma is used here the dilutions have to be made with 1% citrate (see above). The plasma can be inactivated in the usual way (half an hour at 56° C).

Plasma to ½ cc is added to ¾ cc of dilution fluid. With this initial dilution, ¼ cc is measured into each of five tubes. Two of these form the first tubes of the complement-fixation series, the third is the serum control and the other two are the second tubes of the serum dilution series, from which further dilution is carried out. Each series consists of 8 tubes, of which the 2nd to the 8th inclusive contain ¼ cc citrate each. Subsequently, ¼ cc of antigen and the same quantity of complement dilution (two exact units) are added. The racks are left to incubate overnight and the titrations are completed the following day by adding the haemolytic system.

As regards the calculation the following applies. The plasma may be reckoned to contain about a third of the quantity of antibodies

present in the serum of the patient. It was added to the first tube diluted 2 in 5 and subsequently diluted again with the same quantity of virus and the same of complement. Hence the first tube is assessed to possess a titre of 22.5; the second of 45 . . . . . 2280. As we discussed elsewhere in this paper, it proved to be pointless to note down intermediate readings. The last tube with inhibition of the haemolysis is therefore taken as the terminal point.

CHAPTER III

The Serological Material collected during the winter 1948/9

During the winter of 1947/8 we instituted an examination among medical students as to value of the agglutination inhibition test for studying influenza epidemiology (42). Sera were collected at the beginning and at the end of the winter season. It appeared that fluctuations in the results of agglutination inhibition tests were such that no importance could be attached to a twofold difference in titre between the two sera, whereas a fourfold rise or decrease appeared to be significant.

Thanks to the cooperation of the Director, specialists in internal medicine and physicians of Endegeest Asylum, we were able to repeat this experiment on a larger scale. We were provided with a mass of data concerning patients suffering from influenza during the period studied. To our regret we were not able to collect garglings from all patients for virus isolation, since we were often checkmated by the remarkable psyche of a number of patients who only reported sick after they had felt ill for some time. We succeeded in isolating virus from all patients whose clinical symptoms were not older than 24 hours. Two patients caught influenza twice. From one of them, a nurse, we isolated the virus twice.

In November 1948 we began collecting blood specimens from just under 300 patients. The specimens were retained in test tubes. The next day the serum was drawn up into a kind of Pasteur's pipette with a constriction just below the cotton-plug. The tubes were then sealed off at the constriction and at the tip. These "ampoulles" of our own make were marked with a diamond pencil. This simple method made it possible for us to deal with a large number of sera in a short time without great expense and without seriously burdening the laboratory.

The ampoulles were kept at 56° C for half an hour to prevent bacterial pollution, and stored in the refrigerator at 4° C until required.

Blood was collected from all patients immediately after the reporting of symptoms of influenza. This was repeated after 10 to 14 days. Finally we again collected specimens in April, when the epidemic had died down, from all persons from whom this had earlier been done. In this way we had at our disposal serum from 320 persons for the investigation. We executed complement fixation reactions

and agglutination inhibition tests with all these sera concurrently. The reactions were each done with three antigens, influenza B (Lee), influenza A (PR 8) and an influenza A′ strain. The total number of sera subjected to the various treatments was 716. On account of its considerable extent the examination took up a fairly protracted period of time, and there may be objections here and there to certain details of the serologic method. For instance, we omitted pre-treatment of the sera with cholera filtrate, as at the time this method was not so firmly established as it is now (3). The cholera filtrate proved to be of unstable composition. In principle I cannot wholeheartedly approve of this method although I must acknowledge that I cannot suggest a better one. The fact that biological material of unknown composition is added to sera introduces an uncertain element into the experiment. Hence we made use of the method of Briody which has also been described by Van der Veen (4, 36). It appears that in a buffer solution of citric or boric acid the nonspecific fixation of influenza virus gives much less trouble than in physiological saline. We carried out all the agglutination inhibition reactions in a 1% citrate solution. At first we used a mixture of citrate and phosphates adapted to a pH of 7.6, but it later proved to be of no advantage to work with buffer solutions. The results are the same with a mixture of citrate and saline. In this case as well, the pH has to be checked. The mixture we used for the greater part of the investigation was composed of 1% sodium citrate and 0.6% sodium chloride in aq. comm. (pH 7.6).

From numerous preliminary tests it had become evident to us that with both the agglutination inhibition test and the complement fixation reaction better results were obtained when the crude virus suspension underwent a simple purification process beforehand. Centrifuging at high r.p.m. (6000—10000 g) met our requirements best. Other methods tried were adsorption to and elution from red blood-corpuscles; precipitation and resuspension after treatment with methanol, acetone, or ethanol, but they either gave less satisfactory results or were more laborious.

Technically we may describe the methods used as follows: —

A. Preparation of the virus suspensions.

We used PR 8 as A strain, Lee as B strain and Nelemans and Limburg, two strains isolated by us during the epidemic, as antigen for A′. The antigen suspensions were obtained by inoculating 10-day incubated hens' eggs in the allantoic cavity with an appropriate dilution (10^{-5}—10^{-8}) of the virus. For influenza B, 9-day incubated eggs were also used. After inoculation the eggs were incubated

further for two days at 35° C. The live eggs were then brought to 4° C for 2 hours, and harvested. Each egg was checked for titre and bacterial sterility. The allantoic fluid of sufficiently high titre was collected and stored at 4° C.

Shortly before use it was centrifuged for 20 minutes (6000—10000 g). After this treatment the agglutination titre of the fluid was determined for chicken's erythrocytes. Virus suspensions with an agglutination titre less than 2000 were not used.

B. The haemagglutination inhibition test (A.I.T.).

1. Materials:

For both the inhibition test and the titre determination the same set-up was used. For the latter dilution fluid was used instead of serum. The dilution fluid was made up as follows: —

Sodium citrate	10 gr
Sodium chloride	6 gr
Aq. communis	1000 cc

Checked for pH and corrected to pH 7.6 with citric acid and a solution of NaOH.

The chicken's erythrocyte suspension was prepared as follows: Blood was drawn off by heart puncture, and mixed immediately with Alsever's acid citrate glucose solution in the ratio of 2 parts of Alsever to 1 part of blood.

Composition of Alsever's solution: —

Citr. natr. 2 H_2O	8 gr
Sodium chloride	4.2 gr
Glucose	20.5 gr
Aq. dest. to	1000 cc

Bring to pH with 10% citric acid (pH 6.1).

Filter through hard filter-paper and sterilise at 10 lbs.

In the refrigerator the chicken's blood suspension may be kept for a long time. The erythrocyte suspension must always be freshly prepared. We assess that 10 parts of blood suspension (in Alsever) yields 1 part of erythrocytes. In all tests a ¾% suspension (in citrate as above) was used. The final concentration of erythrocytes amounted to ¼%.

2. Fixation.

The inhibition test and the determination of agglutination titre were both carried out in tubes. Equal quantities (0.25 cc) of the three components — virus, erythrocytes, and serum — were used. In defining the titre of the virus we commenced with a tenfold diluted virus suspension (purified allantoic fluid). Of this a dilution series

1, 2, 4, ... was made and 1 part of citrate solution and 1 part of erythrocyte suspension was added. The last tube showing complete agglutination was taken as the final titre of the virus. Originally, interpolated dilutions were also made, so that the final dilution series was 30, 45, 60, 90, 120, 180, ...

However, it appeared that this was of no advantage. Neither was there any point in evaluating incomplete agglutination patterns — it appeared that the values obtained were not distributed along a regular curve due to inability to read the tubes accurately. In executing the virus titrations in duplicate, differences of 1 tube appear to be no exception. For all these reasons we restricted ourselves to the method indicated. The serum titrations were also executed with twofold dilution series of the serum. In that case the final concentration of the virus amounted to 4 agglutination units. By using an automatic pipette (Julius) for making the serum dilutions and an automatic burette ("Hohn's automatic pipette") for adding the constant volumes (virus and erythrocyte suspension) the work was much simplified and it became possible to execute the entire series of titrations concurrently.

C. The complement fixation reaction (C.F.R.).

The C.F.R. was executed with a considerable surplus of antigen, the purified virus suspension being diluted 1 in 10 (corresponding to more than 200 agglutination units). The complement titration took place in the presence of antigen and of a serum known to be influenza negative and Wasserman positive, as well as without these two components. I took this precaution because of the expectation of lues cases with positive Wasserman reactions among the asylum patients. There may be a possibility that lipoids are present in the antigen which might give a reaction with sera from lues patients. However, we found no reactions between our antigens and Wasserman-positive sera.

Preliminary test and main test were executed with equal volumes of serum dilution, antigen, complement, and haemolytic system. For the complement titration the following dilution series was used: 2.5%, 2%, 1.5%, 1.25%, 1%, 0.75%, ... (twelve tubes).

The exact unit of the series without antigen and serum must not differ more than one dilution from that containing both influenza-negative serum and antigen. Two exact units of complement were used for the main test. The complement was either fresh guinea-pig serum or guinea-pig serum that had been kept at —30° C in the deep-freezer for some time. For the haemolytic system we used sheep's erythrocyte suspension freshly centrifuged and washed,

derived from defibrinated sheep's blood. The erythrocyte layer was assessed as 100% and a haemolytic system with an erythrocyte concentration of 1% was prepared from it, the final concentration in the tubes being 0.25%. The haemolytic system contained at least 10 units of amboceptor.

In the main test, three series of twofold dilutions of the serum were made. Here, as in the case of the A.I.T., all sera from each person were treated with the various antigens at the same time. The mixture of serum, antigen and complement was left to react at 4° C in the refrigerator for 16 hours. The haemolytic system used for the preliminary test was also stored in the refrigerator during the same period. Mixtures and haemolytic system were then heated separately in a water-bath at 37° C. The test was then completed by adding 0.25 cc of the haemolytic system to all tubes, mixing well and leaving them to stand at 37° C in a water-bath for at least half an hour. Immediately afterwards the result was read off. At first we interpolated values between complete haemolysis and complete fixation in this test as well. Interpolation, however, again proved inaccurate so that we eventually took the last tube with complete haemolysis as the limit. Two check reactions were always carried out along with the main test, the first being a check titre determination of the complement and the second a check of the antigen used. For the latter a series of twofold dilutions of the antigen was made and a known antiserum in a tenfold titre added to it. Complement and haemolytic system were then added as in the other titrations.

The results.

1. Influenza B.

Thanks to the fact that during the examination practically no influenza B infection occurred we are able to confirm and extend the results obtained during the winter of 1947/8. We had defined the limit below which no value was to be attached to the results obtained with the A.I.T. Now we were able to calculate the same for the C.F.R.

The results are summarised in tables X and XI.

From these results we determined the standard deviations for the A.I.T. and the C.F.R., using log./ratio to convert to linear calculation. In both cases it appeared that no value was to be attached to the ratios 1:2 or 2:1. The chance of this ratio occurring accidentally in the A.I.T. is as high as 1 in 14, and in the C.F.R. as high as 1 in 27. With our technique and our material it was to be expected that a ratio of 1:4 or 4:1 will only occur once in 10,000 A.I.T. observations

and as seldom as once in 100,000 C.F.R. investigations. There is even less chance that ratios outside these values will be coincidental.

TABLE X

The relationship between the A.I.T. titres for influenza B obtained with serum specimens collected at the beginning and the end of the winter season 1948/9

Ratio	Number of paired specimens	Remarks
4 : 1	6	
2 : 1	10	
1 : 1	255	
1 : 2	20	
1 : 4	2	
1 : 8	1	Clin. positive; infl. A negative
1 : 16	0	
1 : 32	1	Clin. positive; infl. A negative

TABLE XI

The relationship between the C.F.R. values for influenza B obtained with serum specimens collected at the beginning and the end of the winter season 1948/9

Ratio	Number of paired specimens	Remarks
2 : 1	26	
1 : 1	259	
1 : 2	18	
1 : 4	1	
1 : 8	0	
1 : 16	0	
1 : 32	1	Clin. positive; infl. A negative
1 : 64	1	Clin. positive; infl. A negative

For instance, a ratio of 1:8 will only occur once in 94 million A.I.T. observations, with a negligable incidence of one in 4,000,000,000 C.F.R. investigations. Hence we may consider ratios of 1:4 or less (4:1 or more) as significant. In the A.I.T. we found significant results with 10 paired specimens. One of these showed a ratio of 1:8, and one a ratio of 1:32. The patients concerned exhibited symptoms of influenza and the test for antibodies was negative for influenza A. We may assume here that they in fact underwent infection with B virus, the more so as the C.F.R. values were also significant. Six pairs of specimens exhibited important titre decreases. Here we probably had to do with cases suffering from influenza B infection before our observation. There remain two cases with a titre rise of 1 in 4. It is possible that there had been some contact with B virus without evincing clinical symptoms. The corresponding C.F.R. ratios

were 1:4 and 1:2. To our regret we did not succeed in isolating the B virus, because the patients reported for medical treatment too late. Remarkably enough, the two cases of influenza B did not give rise to more cases. Perhaps we may explain this by the fact that a fourfold titre decrease was found in 6 patients, indicating that influenza B infections had occurred before. Against this, however, is the fact that on the whole the influenza B titres were fairly low for all sera. The two known infections with B virus occurred at the end of the season. With the data available it is impossible to formulate an authoritative explanation. Of course, one may assume that the virus was not very virulent or that the contacts were immune to influenza B, but we have no evidence to this effect.

This line of reasoning also holds good for the results of the C.F.R. examination. We might have expected a twofold titre rise or decrease in eleven cases, but we found in fact 18 rises and 26 decreases. The latter is particularly indicative of the presence of B virus in the community before the commencement of the investigation.

We also considered whether the data we collected for the B virus might be applied to viruses of the influenza A group. This may not be entirely taken for granted, since there are also other than antigenic differences between influenza A and B. For instance in the laboratory, biological difference is illustrated in the use of older embryos for the isolation of A strains than for influenza B. Epidemiological differences stand out most clearly of all. In point of fact, we know that only A strains seem to cause serious, widespread epidemics, while influenza B gives rise more to scattered, isolated cases. Nevertheless both virus diseases have so much in common that their differences cannot be considered as conclusively against using the data of one for the other. The best evidence for doing this is that the virus A results from the 1947/8 investigation were confirmed by the A.I.T. results for influenza B virus the following year, as described above. Consequently I have taken a fourfold difference in titre as significant for both A.I.T. and C.F.R. with virus A, and have left lesser ones out of consideration.

2. Influenza A group.

We treated the material available in two ways. In the first place we tested the reliability of the various methods of examination, using data obtained from clinical cases of influenza occurring during the period of observation. In the second place we tried to gain an impression of the behaviour of the virus in the community concerned. To this end it was necessary to include sera from patients without clinical symptoms. Finally the data were tabulated to facilitate the drawing of conclusions.

a. Material from clinically positive cases.

In Tables XII and XIII the titre rises less than fourfold are listed as negative results. Strictly speaking this is not statistically correct. Let us take as an example the A.I.T. values obtained with the A strain PR 8 (Table XII).

TABLE XII

Comparison between the frequency of A.I.T. and C.F.R. titre rises in paired specimens of serum from clinical cases

Virus	AIT + CFR+	AIT + CFR–	CFR + AIT–	both reactions negative
A and/or A′	71 (72)	7	7 (6)	2
A	59	7	16	5
A′	64 (66)	8 (9)	10 (8)	5 (4)
B	2	0	0	85

AIT+ or CFR+ indicates a significant rise in titre between the first and last specimens. The numbers between brackets relate to the frequency of titre differences between the two intermediate specimens (see text).

The total number of pairs examined was 87. In 21 cases the titre rise was less than 1 in 4, the remaining 66 being therefore significant. In uninfected material one might expect that 87 results would include 6 of rise or fall 1:2 or more (3 rise and 3 fall). There were in fact 12 with a twofold rise. Hence the conclusion had to be drawn that the other 9 cases were indicative of infection. However, we are unable to tell which of the 12 these 9 cases are. For each of the cases it is true that the chances of their being indicative of infection are 3 to 1. To take another sample, the values from the A.I.T. executed with A′ virus are quite different. Once more, 87 paired specimens were examined, 15 being without significant rise in titre, and 72 with. Here as well, 3 titre rises of 1:2 or more could be expected, but in fact 4 were twofold. In the case of A′ virus it makes no essential difference whether 72 or 73 titre rises are significant.

The numbers are frequencies of significant rises in titre between the paired specimens. At the same time they permit comparative evaluation of the C.F.R. and the A.I.T. For each patient we had at our disposal at least three and usually four specimens of serum. These included the specimen collected in November in most cases, a specimen collected at the onset of the disease, one collected ten days later and the last one collected in April. The numbers in parentheses relate to the two intermediate specimens and are included only when they differ from the rest. The other figures relate to the first

specimen (second, in the absence of the first) as compared to the last specimen.

We observed for the A′ virus one single non-significant C.F.R. titre rise from the first or second specimen to the third. Apparently the titre rise was not yet complete at the time, as we read off a fourfold difference in titre as compared to the last specimen.

The A.I.T. results exhibited somewhat larger fluctuations, the largest being those for A′ antigen. We originally found in three cases a difference between the first and third specimens less than fourfold and later (first and fourth specimens) significant titre rises. On the other hand there were three cases where the initial 16, 8, and 4 fold titre rises were reduced to twofold. These are comprised in the figures between brackets in Table XII, and effect a further slight reduction in the difference between A.I.T. and C.F.R. results.

From this we may conclude that for confirming influenza on serological grounds the two methods are of equal value. The influence of the antigen upon the results shows up more clearly in the A.I.T. than in the C.F.R. Nevertheless the differences are not so clear as to indicate the use of the two methods. If we calculate the standard deviations (S.D.) for the percentage of positives obtained by the two methods we find S.D. = 6.35 (antigen A). The difference amounts to 6, which is 0.9 times the S.D. The chances against this difference being coincidental are only 1.75 to 1 (calculated according to the Van der Waerden correction to the standard deviations of percentages formula).

TABLE XIII

Comparison between the results obtained with influenza antigens A and A′

reaction:	A + A′+	A + A′—	A′ + A—	negative with both antigens
A.I.T.	60 (62)	6 (4)	12 (13)	9 (8)
C.F.R.	71	4	3	9

A+ or A′+ indicates a significant rise in titre between the first and last specimens of the pair with influenza antigen A and A′ respectively. The numbers between brackets relate to the frequency of titre differences between the two intermediate specimens (see text).

From Table XIII it is evident that it is of no importance in the C.F.R. which of the two antigens is used. This is not so in the case of the A.I.T., where the difference between the values found for A and those found for A′ is 9. The S.D. of this difference is 5.64, that is to say that the chances against its coincidental occurrence are 8 to 1.

The statistical arrangement of the data serves to demonstrate

that we cannot draw any convincing conclusions from them, as there are not yet enough of them. At the same time, the investigations serve to underline what was already known from other sources i.e., that the C.F.R. is a reliable method, which, however, does not enable one to distinguish between the finer differences in composition of the antigen (33). The question whether a certain case is an influenza virus infection or not can be solved at least as well with the C.F.R. as with the A.I.T. It is probable that better results would have been obtained by the latter method if we had applied cholera filtrate treatment (with the appropriate antigen A′). The advantage of simpler execution would, however, have been cancelled out by the pre-treatment complication. Moreover a distinct difference should be made here between the diagnosis of a single case and the study of an epidemic. In the latter case we know the type of virus concerned and may choose the most applicable antigen. When the strain is unknown, the C.F.R. is preferable to the A.I.T.

b. Investigation of the material as a whole.

The results of the examination of specimens from all the inhabitants of Endegeest Asylum, including those without clinical symptoms of influenza, lead us along a different path. Of the 320 persons whom we had included in the investigation, 26 dropped out for one reason or another, leaving us with data concerning 294 persons. The patients included in this total belonged to a closed community. But there were a number of nurses working in the wards concerned, and they formed one of the links with the world outside.

The first cases of influenza occurred in December 1948. About the same time one nurse and a patient working during the day-time outside the ward fell ill with typical symptoms of influenza. From both of them the virus was isolated and found to belong to the influenza A′ group. The nurse worked in the men's ward to which the patient concerned belonged, and it was not before February 1949 that a new case of influenza was diagnosed in the same ward.

In January 1949 the first cases occurred in the women's ward. In Table XIV below, the dates on which the various cases were reported are listed consecutively.

Dividing the material into three groups — nurses, male and female patients — produced no information as to the route followed by the infection. Observations taken indicate that the period of influenza incubation need not be much longer than 24 hours (I myself fell ill in 1947 36 hours after a laboratory infection). It is not clear whether such a short period of incubation is the rule or the exception.. From Table XIV no data can be derived from which we are able to calculate the period of incubation. The somewhat

higher numbers of cases reporting on Mondays can be explained in various ways, none of them clearly predominating. Perhaps some of the cases actually commenced on the preceding Sunday. On the other hand it would be possible that visitors introduced the influenza virus on Sunday and that the patients fell ill 24 hours afterwards.

TABLE XIV

Cases of influenza by date of report of the first morbid symptoms

Date	Number	Date	Number	Date	Number
Dec. 31st	2	Jan. 30th	1	Febr. 9th	2
Jan. 2nd	3	Jan. 31st (M)	14	Febr. 10th	3
Jan. 3rd	2	Febr. 1st	2	Febr. 13th	2
Jan. 10th	1	Febr. 2nd	3	Febr. 14th (M)	10
Jan. 21st	2	Febr. 3rd	1	Febr. 16th	5
Jan. 24th	3	Febr. 4th	2	Febr. 17th	8
Jan. 25th	2	Febr. 5th	4	Febr. 18th	5
Jan. 26th	1	Febr. 7th (M)	9	Febr. 23rd	1
Jan. 27th	3				

(M) = Monday

Against this is the fact that most of the Monday reports came in during the morning, indicating that the patients would have to have been infected on the Saturday. Upon inquiry, is was found that most of the cases had no Saturday visitors. Another possible explanation would have been a closer contact between patients during the weekend. But in fact many patients were in close contact throughout the week in spite of ample compliance with hygienic standards in the dormitories and rooms at Endegeest (space, ventilation). Although this does not sum up all the possible explanations of the higher number of cases on three successive Mondays, I am of the opinion that no point is served by going deeper into the matter.

It did not appear possible to learn anything further about the epidemiology of influenza A′ by merely studying the clinical cases. However, we also obtained sera from other persons in the community. Perhaps I may mention here that we did not succeed in 50 attempts in isolating the virus from these latter persons. In Chapter II we explained how we looked for alternative methods to egg inoculation for indicating virus. If we had succeeded, we would have conducted serial examinations of a much greater number of individuals. In the absence of such methods we were only able to put part of the material to good use (limited tenability of the virus at —30° C, laborious technique, restricted supplies of eggs etc.). We had to be satisfied with serological examination.

TABLE XV

Comparative serological examination of influenza (A) in 294 inhabitants of Endegeest Asylum, 1948/9

I	II	III	IV	V	VI
clinical	titre rise 4 or morefold	titre rise 2 or morefold	titre rise significant	no rise in titre	total
positive	87 (89)	87 (89)	87 (89)	2 (0)	89
negative	83	150	144	61	205
total	170 (172)	237 (239)	231 (233)	63 (61)	294

The figures in brackets are corrected for the assumption that two persons suffered from influenza B.

According to calculations already mentioned we might expect in non-infected surroundings that a titre rise or fall of 2 or morefold would be found in 1 case in 27, or 11 in 294 (Table XV). Hence we might expect 6 rises, which are deducted from 237 (total column 3) leaving 231 as the number of titre rises caused by contact with influenza virus. These corrected figures are inserted in column 4. The figures speak for themselves, Of the 294 persons concerned in the investigation 89 were clinical cases. Of the remaining 205 we may assume that 144 underwent contact with influenza virus without exhibiting clinical symptoms. More than 20% evidently escaped infection, though there is no certainty at all that mere failure to demonstrate antibodies excludes contact with the virus.

One interesting point became evident during the course of the investigation. In the C.F.R. we found, with two exceptions (9 and 18) an initial titre of less than 9 (immeasurable) for the clinical cases. As regards the negatives, we distinguished two groups — 150 patients with titre rises and 55 without. In the first group we found in all but one (9) a titre of 18 or higher for the first serum. In the second group an initial titre of 9 or lower was found 16 times (out of 55). We are of the opinion that some value should be attached to a low initial titre. It is very tempting to draw the conclusion that 16 of the group in which no clinical symptoms of influenza were found would have become clinical cases if they had come into contact with the virus. We know, however, from experience of other contagious diseases that serological data may never be taken as absolute criteria. It may happen once in a while that contact with infectious material and even the breaking out of morbid symptoms is not followed by an increase of demonstrable antibodies in the blood.

It was not possible to draw any conclusions from the high haem-agglutination inhibition titres of the first specimens. We are of the

opinion that this cannot be a consequence of omitting pre-treatment with cholera filtrate. We know from our own experience that even with this treatment high initial titres occur in specimens from influenza patients.

It has already been mentioned that one of the nurses exhibited typical influenza symptoms twice.

The titre values obtained for this particular case by the various methods of investigation are summarized in Table XVI.

TABLE XVI

Serological data in case no 289 (nurse suffering twice from influenza)

I	II	III	IV	V
Date	C.F.R.		A.I.T.	
	A(PR 8)	A′(own strain)	A(PR 8)	A′(own strain)
31 Dec. first accute phase	—	—	72	48
10 Jan.	9	9	288	384
16 Febr. second accute phase	9	—	576	1536
26 Febr.	72	1152	4608	12288
? April	54	576	576	6144

— = < 9

It may be that Table XVI is another argument for the use of the C.F.R. as a means of measuring the degree of immunity. However, it does not seem justified to lay too much emphasis on conclusions drawn from one case only. In all cases from which virus was isolated, the serum specimens were tested with their own virus strains. The A.I.T. titres of a number of these specimens were slightly higher with their own strains than with one of the other A′ strains. Mostly, however, the difference was not large enough to lead to conclusions. In a case from which the "Stockholm" type of A′ strain was isolated, the patient's own strain produced considerably lower titres than the strains of the "Liverpool" type used in the investigation. The variations were considerably less with the C.F.R.

At this stage we may draw several conclusions. To begin with, it is clear that the virus, in spite of its appearance in a community living in such close relationship, has only progressed slowly. It is possible that a high initial immunity condition of the population was the reason. It is evidence for this, that a great number of inhabitants of Endegeest Asylum demonstrated antibodies as measured by C.F.R. at the very beginning of the investigation.

It is not clear whether the influenza epidemic was caused by a single or a multiple introduction of the virus into the community. This subject is developed further in the next chapter.

It became quite evident from our results that the clinical examination of influenza patients is at least as well served by the C.F.R. as by the A.I.F., if not better. In my opinion it is advisable to apply the C.F.R. as a routine check on clinical diagnosis. The A.I.T. in its present form with cholera filtrate pre-treatment is every bit as complicated as the C.F.R. The greater specificity of the A.I.T. turns to a disadvantage when it is not known which strain type to expect. As the C.F.R. is less sensitive to the type of the strain, fewer cases will be missed, though the type of the infecting strain cannot be identified. Moreover, the C.F.R. is heir to less inconvenience from the lower antibody production of such strains as the "Stockholm" A′ virus.

It is interesting in itself that the introduction of a virulent influenza strain (one of the patients died of post-influenza complications) into a closed community does not lead of necessity to an explosive outbreak. We may assume that this is a result of the relatively high degree of collective immunity. Vaccination should produce a comparable situation. Investigation into the benefits of vaccination would be able to make use our data. We know that vaccination is nearly always followed by a significant rise in circulating antibodies. Evaluation of antibody titre before and after influenza epidemics could teach us a great deal about the effects of vaccination, about the duration of protection, the immunity percentage, the number of infections occurring despite vaccination, etc. In my opinion it would be justified to conduct a vaccination experiment in similar surroundings to those described in this chapter, using more or less the same technique.

CHAPTER IV

The examination of the virus strains isolated during the influenza epidemic of 1948/9.

The thirty virus strains isolated during the 1948/9 epidemic from patients at Endegeest Asylum were subjected to close examination. They all appeared to belong to the A′ group. The investigation might have ended there, but for the differences occurring in this group, the meaning of which is not as yet clear. Strangely enough, a good deal is written about differences in antigenic composition among these virus strains. The use of the term "antigenic difference" is only justified as a result of an observed qualitative difference in reaction to antisera, demonstrable either by absorption tests or by reaction to antibodies against purified antigens. Quantitive differences — differences in titre — are not sufficient. An example of an analogous case of titre difference may be found in bacteriology.

There are strains of Salmonella typhi that are well agglutinated by group D serum, while others possessing the same O-antigen are not agglutinated at all by it, since they are covered with well developed Vi antigen. Still other strains exhibit intermediate titre values. Another example is the behaviour of Brucella strains. We know of at least two antigens, both found in Brucella abortus Bang, Brucella abortus Suis and Brucella melitensis — the A and the M antigens. These strains exhibit difference in titre when reacting with anti-Brucella sera, but this does not justify the conclusion, that there is a difference in antigenic composition (40). Neither of these examples is exactly analogous to the behaviour of the influenza virus. For instance strains of the A′ "Stockholm" type have low A.I.T. tifres and at the same time appear to be incapable of stimulating the production of high value antiserum in ferrets. In bacteriology a similar behaviour does not seem to have been observed.

This line of reasoning led us to carry the investigation of the isolated strains further. We were also interested in coming to a conclusion as to whether or no "Liverpool" strains can evolve into "Stockholm" type strains and vice versa. A negative conclusion on this point would indicate multiple introduction of virus during the course of the epidemic. The accumulation of new cases on Mondays would seem to support multiple introduction (see Chapter III). The closed community and the generally restricted contact with visitors, however, are evidence against this. The absence of a really explosive

outbreak despite close contact between the patients must be ascribed to other factors, in my opinion. Probably the generally good state of health and nourishment played a part here. It is not impossible that absence of other infections may have been of assistance.

Van der Veen and Mulder (37) have pointed out that there are three different types of A' strains, which they called p, q and r at the time. Nowadays we usually refer to the "Stockholm" and the "Liverpool" types. The third type is generally not named separately as it exhibits characteristics intermediate between the first two. The "Liverpool" type is known to create a potent antiserum in ferrets, and is inhibited by its own antiserum up to high titre values in the A.I.T. The "Stockholm" type, on the contrary, is not capable of creating a potent antiserum in ferrets and the serum only reacts with "Stockholm" or "Liverpool" strains up to low values in the A.I.T. The "Stockholm" strain is only inhibited up to low titre by potent "Liverpool" antisera. In my opinion it is dangerous to set too much store by whether or no a certain strain is capable of creating large quantities of antibodies in the laboratory animal. This may be dependent on the virulence of the strain without necessarily involving differences in antigenic composition. Quite apart from this capacity, A.I. Tests with one and the same serum disclose differences according to whether they are carried out with "Stockholm" or "Liverpool" type strains.

One may advance two explanations: 1) the dose of virus used is larger in the case of the "Stockholm" strains so that the difference is not a true one (i.e. there is more virus present per agglutination unit); 2) the virus particles are encapsulated (cf. the Vi antigen in Salmonella typhi) which renders them resistant to haemagglutination inhibitive antibodies.

In 1) there is no question of a difference in antigenic composition. It is not easy to account for the fact that this implies the virus has less antigenic capacity. However, this need not lead to rejection of this assumption as improbable. In 2) there may exist a difference in antigenic composition. Consider for instance the case in which one strain is encapsulated and the other not. But it may be that the encapsulating antigen is present in both strains. In that case, it may operate as a check to other antigens in one strain and not affect them at all in the other (cf. the Vi antigen in Salmonella typhi).

The whole question becomes still more complicated when we remember that it has proved possible to convert strains of the "Stockholm" type into strains which come very close to the "Liverpool" type. The simplest manner is to pass the strain on to another species of host, for instance from the egg to the mouse and subsequently (after a number of passages) back to the egg. Here as

well, one may trace the analogy to the Vi antigen in typhoid bacteria. When inappropriate substrata are inoculated with Salmonella the strain easily loses its power of producing abundant quantities of Vi antigen.

At this stage, it will be profitable to go a little deeper into the effect of passage through various hosts on virus strains. Contrary to bacteria, which are freely living organisms, the influenza virus like other virales cannot multiply other than in the living cells of a host. There is every reason to assume that in transmission from host to host there arise changes in the composition of the virus, possibly amending its antigenic properties. One would expect the effect to be accentuated when the hosts concerned belong to different species. There are striking examples of this in yellow fever and smallpox virus.

Burnet (6) has shown us how changes occur in the influenza virus from man when it is isolated in eggs. The original (O) virus from the first egg passage is unable, or hardly able, to agglutinate chicken's erythrocytes. In the ensuing passages the virus derivate (D) becomes able to agglutinate erythrocytes up to high titre values. The O virus is capable of reacting with human erythrocytes, a property which remains more or less preserved in the ensuing egg passages. Earlier we proved that the capabilities of agglutinating these two sorts of erythrocytes occur independently of each other and must be due to different antigens (14). There is no reason to suppose that the passage from one species to another would always follow the same pattern. Hence in my opinion, it is not impossible that there is in nature only one type, which our laboratory procedures invest with the characteristics known as those of the "Liverpool" and "Stockholm" types.

The fact that in some epidemics the first type occurs almost exclusively and in others only the second, does not rule out this supposition. In the epidemic described in the preceding chapter we came across strongly marked examples of both types and in addition a great number of intermediate strains. Of the thirty strains isolated during the 1948/9 epidemic from patients of Endegeest Asylum two belonged to the "Liverpool" type, six exhibited marked properties of the "Stockholm" type, while the remainder were somewhere between the two.

Experiments aimed at investigating this subject were subsequently conducted. The cholera filtrate for treating the sera was put at our disposal by Prof. Mulder and Dr Brans, and we followed the treatment technique applied by them (3).

We obtained the sera from ferrets, which we infected intranasally

with the virus isolated in eggs. Blood was drawn off before the infection and again 14 days afterwards.

The initial dilution for the A.I.T. was 18 as we had to dilute the sera 6 times for the treatment with cholera filtrate. The dilution in the last tube showing complete inhibition when multiplied by four (virus concentration 4 agglutination units) was taken as the titre of the serum. The high initial dilution probably accounts for the fact that the six obviously "Stockholm" type strains were not fixed at all by the "Liverpool" serum. The titre of this last serum with respect to its own strain and to the two other "Liverpool" strains was 9216. The agglutination of other strains was inhibited by this serum at titre values from 144 to 2304. The "Liverpool" strains reacted up to a titre of 1152 with a "Stockholm" type antiserum. However, the strains classified by us as "Stockholm" types were inhibited by this serum up to a titre of 288. The titre of the other strains varied from 144 to 1152 with one exception, which was inhibited up to a titre of 2304. As this strain was inhibited further than the "Liverpool" strains we paid special attention to it in subsequent tests, when it appeared that the titre for both "Liverpool" strains and for the exceptional strain (Khaut) was 1728.

We see here the remarkable occurrence that the "Stockholm" strains were inhibited by their own serum but not, or at least up to a lower titre, by the "Liverpool" serum. Moreover a number of the strains not reacting well with the "Liverpool" serum were inhibited strongly by the "Stockholm" type serum. Both results may be explained by assuming the "Stockholm" strains to possess an antigen not possessed by the "Liverpool" strains.

The investigation was executed with the A.I.T. and though indications have been obtained that the difference in behaviour may be caused by a difference in antigen composition, we may not assume this to be a fact without further examination. In a number of simple experiments Salk (33) demonstrated that extremely small differences in antigen composition are shown up by the A.I.T.

From our own work on avidity (14) it is clear how intricate the antigenic composition of the influenza virus must be. We have to do here with a number of antigenic components capable of fixation by the same antibodies, which react differently as regards the agglutination of erythrocytes. It will be clear that additional evidence is needed to prove the existence of a true difference in antigenic composition between the "Stockholm" and "Liverpool" types. This we attempted to procure by means of the C.F.R.

Generally the C.F.R. is a method of investigation for proving similarities, rather than small differences. Here we made use of it

in order to be independent of the quantity of virus relative to the serum titre.

First we determined the minimum dose of virus suspension that produced fixation with an overdose of serum. Then we determined the titre of the sera using ten times the minimum dose. Remarkably enough the results of this experiment differed from what the A.I.T. results had led us to expect (see Table XVII).

TABLE XVII

Comparison between "Liverpool" and "Stockholm" type strains using the C.F.R.

Strain	"Liverpool" serum	"Stockholm" serum (Prucha)
Liverpool	1152	576
Khaut (type L)	1152	576
Prucha (type S)	288	288
Limburg (type S)	288	288

Ten times the minimum dose of antigen as determined in the C.F.R. was used in every case.

Table XVII contains a random selection from the many titrations carried out. The titre relationships are constant, not being altered by pre-treating the sera with cholera filtrate. One might feel justified in drawing the conclusion from these results that "Liverpool" strains possess an antigen missing in "Stockholm" strains.

At all events it is evident that the quantity of virus in the suspensions cannot have had any influence on the results, as we made sure beforehand that we were using a surplus. We are forced to assume that there is a difference between the two types, though these experiments do not indicate why "Liverpool" strains react better than "Stockholm" strains. Are we to assume that the only difference between the two is that one is more easily and better fixed than the other by the appropriate antibodies? May not the same phenomenon perhaps also be the cause of the difference in antigenic action? Here again the question of encapsulation turns up. Is there perhaps present some matter, either acting antigenically or not, which shields the virus particles, blocking the reaction with the appropriate antibodies and checking their production?

These questions will remain unanswered until we succeed in demonstrating indisputable antigenic difference by means of adsorption tests, on the lines of those carried out by Castellani on Salmonellae. Fortunately many courses are open to us in our search for a solution to this problem. We have already explored several of them, but so far without result. We have attempted, for instance,

to purify the virus by centrifuging, adsorption to erythrocytes, and by chemical treatment (ethanol, di-ethylether), and to reduce it to its components (23, 38). Preliminary tests in this direction however are not very promising, and we have not yet been able to solve the problem we had set ourselves. We have not proved that there exists a difference in antigenic respect between the strains of the "Liverpool" type and those of the "Stockholm" type. But neither have we been able to exclude it.

For these reasons it has been impossible to prove that the remarkably long course of the influenza epidemic in Endegeest Asylum was caused by repeated introduction of the influenza virus. Nor has it been proved, on the other hand, that we had to do with one single introduction of the virus into a closed community.

CHAPTER V

The development of the influenza virus in the (broncho-epithelial) cells of the lung of the mouse.

In the introduction I explained that the study of the virulence of infectious agents should form a part of epidemiological research. The preceding chapter was an attempt to provide an insight into the differences in antigenic composition between influenza strains. In this chapter we will discuss the examination of certain of the biological properties of these strains. The quantitative approach assumes that it will be possible in further investigations to use the results here mentioned for determining the virulence of influenza virus strains. The object of the experiments was to learn something about the behaviour of the virus within the living cell, but the subject is also one that fits in with the treatise as a whole.

There are serious gaps in our knowledge of virales. We are able, it is true, to demonstrate a "virus" and to identify and follow it on its way from host to host, but we are not aware of what takes place within the host or its cells. There are two opposing opinions: those who consider the virus as a living organism with an individual existence (15), and those who consider it to be inanimate (28). There is a good deal to be said for both, and in my opinion it is impossible to take sides in the matter, because of insufficient information. Moreover the facts we do have, concern virales that differ widely from each other in both quality and choice of host. Arguments which may apply to one virus cannot be applied to another as a matter of course.

Investigations into the behaviour of T coli bacteriophages within their host cells (8) are fascinating. Those who consider the virus to be inanimate point out that, when these bacteriophages penetrate into the bacterial cell, the particles leave their images. From the moment of penetration the metabolism of the host is changed. During the first 7 minutes protein foreign to the bacterium is formed, the protein of the bacteriophage. After that the build-up of nucleic acid (desoxyribose-nucleic acid) begins. This phase lasts until about a quarter of an hour after the infection of the cell. During this period it is not possible to demonstrate bacteriophagic particles in the bacteria either by means of electronic micrography or by infection tests. At the end of the period the synthesis of the covering substance begins.

Those who consider the virus to be a living organism point to the fact that from that moment the number of bacteriophages increases geometrically (1, 2, 4, 8, 16. . . .) (15). They argue that the production of bacteriophages by means of the mechanism of the bacterial cell would result in an arithmetical progression (1, 2, 3, 4, 5, 6, 7, ...) The division of the bacteriophagic particle as an independent organism availing itself of materials it induces in the bacterial cell, would follow a geometrical progression, as we in fact perceive in the case of bacteria. They overlook the fact that during the first 14 minutes after infection no bacteriophagic particles are demonstrable in any way. That the bacteriophage loses its image at the moment of infection is also unimportant in their eyes; they point out the behaviour of spermatozoids in fertilization, where an important part also remains outside the ovum.

Neither group has given up the fight by far. Genetics has been pressed into service. Phages that possess peculiar properties are crossed with each other; research is being conducted on phagic particles made deficient by ultra-violet radiation etc. In consequence of all this, higher mathematics have been introduced by the authors concerned, with deplorable results. For instance, two articles appeared on one series of experiments, the mathematical development of which purported to produce diametrically opposing conclusions (15, 29).

The protagonists of "virus as organism" (5) would possibly have a weak case, were it not for the similarity between the increase of virus particles and the "transformation" phenomenon as described for Streptococcus pneumoniae and other microorganisms (1, 13). Pneumococci may form capsules *in vivo*, but they usually lose this property *in vitro* unless special precautions are taken. With the aid of extracts of pneumococci capable of forming capsules, it appears possible to induce pneumococci that have lost this property to regain it. The substance responsible for the transformation is a polymer of desoxyribose-nucleic acid (D.N.A.). The power of forming capsules can be retained throughout repeated passages. By means of serologic reactions it is possible to distinguish a large number of antigenically differing capsules. The capsule type of the pneumococcus from which the extract is prepared defines the capsule type of the transformed pneumococcus independently of the capsule type it previously formed. The transformed cocci may in their turn provide extracts for transforming further cocci. It is interesting that the substance responsible for transformation is a D.N.A. polymer (21). Everywhere in nature where there is transmission of hereditary properties, these appear to be bound up with D.N.A. In fact chromosomes exist for the greater part of this substance. We may reasonably assume

that the phenomenon of transformation also occurs in nature.

The analogy to virus production lies in the principle of auto-reproduction. Besides bringing about change in the metabolism of the pneumococcus as regards capsule formation, the D.N.A. polymer induces the formation of more D.N.A. polymer identical to itself. Surely this is exactly what one would expect of a filtrable virus. In practice, it is true, the host cell is often destroyed after infection with the virus, but this need not always happen.

There are other examples of this sort of analogy, one of the more striking being Bittner's "milk factor" (2). In some stocks of mice the females may be affected by malignant breast tumours in later life. Extensive investigation brought to light that proneness to breast cancer is transmitted to the daughter through the mother's milk. The authors (19) succeeded in separating this factor from the milk and purifying it. Once more, the substance produced proved to be a D.N.A. polymer, which without doubt closely resembles our conception of the filtrable virus. The work of Bittner et al. initiated the development of a theory concerning cancer as an infectious disease — the so called "vertical transmission of cancer" (20). Apart from the problem whether this particular transmission may be considered analogous to virus infection, it is worth underlining how important the part of D.N.A. is in all these processes. It is found in all living organisms and in all filtrable viruses.

The question of whether virales are to be termed animate or inanimate is in my opinion independent of whatever solution turns up for the problems discussed above. Surely it depends purely on the definitions assigned to the words "animate" and "inanimate". When one considers the phenomenon of auto-reproduction as decisive in these definitions, the "milk factor" and the transforming agent of the pneumococcus must be seen as "animate". But when one demands the fulfilment of further conditions, there is quite a chance that a fair selection of "organisms" would have to be excluded from the concept "animate". Many investigators are inclined to dismiss the problem of life and death altogether as insoluble as regards filtrable viruses, and to come to the conclusion that in this borderland no sharp line between "animate" and "inanimate" can be drawn.

Apart from problems of this type, it is important to learn something of the behaviour of the virus within the cell. I would like to mention here a few of the investigations in this field.

Hoyle (22) has studied the course of infection with influenza virus in the membranes of incubated hens' eggs, making use of refined virological and immunological techniques. He has shown that there is a period commencing shortly after infection during which hardly any virus can be demonstrated, either in the extra-embryonal fluids,

in the egg membranes, or in the embryo itself. About two and a half hours after the inoculation of the eggs he finds the first symptoms of virus infection. At that moment haemagglutination appears and slightly later the complement fixation antigen. The complete virus does not appear in the extra-embryonal fluids until a later stage as may be proved by means of infection tests or the C.F.R. What happens within the cell is not disclosed by these experiments.

Here the possibility is opened up for the comparative examination of influenza strains. Independently of the (indeterminable) number of virus particles causing the infection, we are able to measure the rate at which the virus increases (or is increased). Actually we can measure two entities: —

a. the time intervening between the infection and the earliest moment at which the virus (or haemagglutinin) is again demonstrable;

b. the ratio between the quantities of virus (or haemagglutinin) demonstrable after certain given intervals.

When, in addition, we desire to know what takes place within the host cell, other procedures are indicated. As we know that D.N.A. is an integral component of the virus, it is rational to look for a method by which the metabolism of this substance can be studied quantitatively. The Swedish investigator Caspersson (7) has been engaged in the study of nucleic acid for a long time. He makes use of the fact that these acids show an absorption line under radiation at a wavelength of 2560 Å. Working with monochromatic light of that wavelength, and a quartz micrographic apparatus especially devised for him, he has been able to obtain an impression of the metabolic changes in nucleic acids within the living cell. With his method it is not possible to distinguish directly between ribo and desoxyribo-nucleic acids. This last can only be achieved by causing enzyme preparations to act upon tissues. Criticism of the methods used by Caspersson and co-workers is aimed partly at his use of enzymes. However, there are also objections to the technical design of the apparatus which is used for the examination. Moreover the apparatus is exceedingly expensive, both in first cost and in maintenance. Nevertheless, if no other method had been available, we would have had to make use of it, though we would probably have replaced the expensive quartz lenses by a mirror optical system. The quartz monochromator might have been replaced by interference filters. The search for other means has been attended with a measure of success. These also have their disadvantages. Caspersson used unfixed sections while the others use fixed preparations. At first sight fixation seems to be a disadvantage but the unfixed material

has to be treated immediately, rendering it difficult to make large series of observations with the same preparations, and more or less excluding the treatment of the considerable numbers of sections necessary for our investigations. These are by no means all the arguments against Caspersson's method, but there is no point in going further into it, as we made use of another method.

For a long time now the so-called Feulgen reaction with Schiff's reagent has been used for demonstrating D.N.A. The aldehyde groups in the D.N.A. molecules are set free to react by HCl hydrolysis, and then reacted upon by leukofuchsin, whereby the colourless leukofuchsin is converted into fuchsin. The surplus of leukofuchsin is then removed by treatment with SO_2. Several investigators have pointed out that the colour intensity of the fuchsin would be proportional to the quantity of D.N.A. present in the preparation treated. The red colour is caused by the fuchsin absorbing light of other wavelengths e.g. green.

The Feulgen reaction may also be used for staining D.N.A. in tissue. There are a number of types of apparatus for measuring the intercellular absorption intensity of green light (27, 34). Opinion is still divided about the specificity of the Feulgen technique for demonstrating D.N.A. in tissue sections. Stowell (34) and Danielli (10) have brought the pros and cons to the fore. The latter pointed out that aldehyde groups may be freed for reaction from other substances in the tissue than D.N.A. by HCl hydrolysis. Stowell on the other hand showed that, though a theoretical possibility, in practice the effect of aldehyde groups other than from D.N.A. is small enough to be disregarded. However it is only of secondary importance for the present purpose whether the absorption values measured are based on the presence of D.N.A. or other substances formed under the influence of virus metabolism. To view the following critically it is necessary to bear in mind that one cannot be sure whether the changes observed in the absorption values of the preparations tally with the changes in their D.N.A. content, but this is of no great importance for the purpose in hand. In the first place our object was to find a method for determining changes in the cell contents resulting from virus action. In the second place we hoped to learn something about the sites at which the various processes take place. It is only in the last place that the question arises as to whether the changes observed are based on variations in the D.N.A. Nevertheless one must not conclude that measuring the absorption of green light in Feulgen-treated preparations is of no importance for the investigation of D.N.A. in the cell. Even Danielli cannot deny that the fuchsin-red colour in tissue treated by the Feulgen technique must be largely due to the presence of thymo-nucleic acid (D.N.A.).

We selected Professor Lison's apparatus. It not only has the great attraction of being simple in use, but also possesses advantages from a physical point of view. The most important of these is that it only employs the central beam of the microscope and therefore requires no correction for the rest of the visual field.

In principle Lison's apparatus works as follows: The Feulgen-treated section is micro-projected on to a screen using the apochromatic oil-immersion objective. The screen has an opening concentric with the longitudinal axis of the microscope. Behind the opening is an electronic amplifier, and a green filter is interposed to restrict the light passed to a wave-length absorbed by fuchsin. The intensity of green light passed through is measured by the amplifier. The quantity of light absorbed by the fuchsin can then be calculated. In this case the fuchsin concerned is that contained in a cylindrical section of the tissue. To determine the absorption of the whole of an object to be measured (e.g. a cell nucleus), one has to relate the value measured to the volume of the object. In the case of a nucleus, one may say that this value is proportional to the thickness of the nucleus parallel to the light rays multiplied by the density of fuchsin. The remaining factor in the quantity of fuchsin is the projected area of the nucleus, as seen on the screen. The outline of the projected area can be copied on paper and measured by a planimeter. The amounts of D.N.A. in the respective nuclei, relative to each other, are found by multiplying the area of each nucleus by its absorption value.

A more detailed description of this method is given in Lison's own publication (27).

In order to acquaint myself more fully with this method of investigation, I worked with Professor Lison's apparatus under his guidance (in Brussels at the time). From the preliminary work done there it was established that egg membranes were unsuitable for the investigation. In the Feulgen-treated embryonal tissue of the chorion allantoic membrane it is not possible to identify with certainty the various kinds of cells present, which seriously reduces the chances of success. Other possible materials were the nasal mucous membrane of the ferret and the pneumo-bronchial epithelium of the mouse. Although we carried out some experiments with the former, we finally changed over to the latter mainly because it was more readily available in quantity. Moreover it had the advantage of being amenable to Hoyle's set-up. This combination of material and method enabled us to study the development of influenza antigen and infectious virus particles, in addition to our first object of investigating virus metabolism in the cell.

Materials and methods.

Influenza strains: W.S. Prof. Mulder was good enough to make a supply of this strain available to us. The exact formula of the number of passages was not known, but we do know that the strain was passed via ferret and mouse to eggs. It was passed intranasally to mice by us and underwent 6 mouse passages before the main experimental series was set up.

PR8 X. This strain was put at our disposal by Dr. van Tongeren. Up till now only infection tests and antigen titre determinations had been executed with it, the reason being its extraordinary virulence in eggs. The embryos of eggs inoculated with PR8 X generally die within 48 hours. The strain produces a haemolysin contrary to our reference strain PR 8.

Eggs and animals. The hens' eggs were white Leghorns with the exception of those used for the test with PR8 X virus, which was partly executed with "Noord Hollandse Blauwen" eggs. The eggs were pre-incubated at 39° C (102° F) for ten days, and inoculated in the allantoic cavity. Two days after inoculation the eggs were harvested and the allantoic fluid tested for agglutination with a suspension of chicken's erythrocytes (4 drops of allantoic fluid to 1 cc ¼% erythrocytes suspension). One drop of allantoic fluid was always added to glucose broth for checking bacterial sterility.

The mice originated from our own stock. They are the so-called Swiss mice inbred to produce a pure breed. We mainly used mice of 12 to 15 grammes and about as many males as females. For one experiment we bought mice from an animal dealer who originally started breeding with animals from our own stock.

The mice were intranasally inoculated with 0.05 cc suspension under ether and chloroformed 24 to 36 hours after infection. The lungs were prepared under sterile conditions, and those used for re-inoculating were rubbed fine with sand in a mortar, adding 10 cc of a mixture of broth, saline and antibiotics. The mixture consisted of equal parts of saline and broth (bacteriological) to which penicillin to a final concentration of 400 units per cc and dihydrostreptomycin to a final concentration of 0.25 mgr/cc were added. The dilutions were also made with this mixture. The lung suspension so prepared was assessed to be 10% (10^{-1}).

Lungs from which microscopic specimens were prepared were very carefully treated without touching the lung tissue. For the research mentioned below they were fixed in the following mixture: formol 40% 10 parts, radical vinegar 10 parts, ethanol 90% 80 parts.

For the preparation of the specific antisera we used ferrets from our own stock, infected intranasally under ether. Before and after

the infection, blood was drawn off by means of heart puncture. A.I.T. and C.F.R. tests were carried out with the same antigen, with antigens of other influenza strains, and with a normal allantoic fluid as a check.

Methods of investigation.

For the determination of the infection titre of virus suspensions we made a series of successive 10-fold dilutions (10^{-1}, 10^{-2}, 10^{-3} etc.) using the broth-saline-antibiotics mixture. Each dilution was introduced into the nasal cavities of four mice.

The mice were chloroformed 4 days after infection, and during dissection the extent of the lung lesions was established as follows:

Virus pneumonia in a small part of one or two lobes was indicated by "+";
a number of spread foci in two or more lobes or confluent lesions throughout an entire lobe "++";
involvement of an entire lung and a single small focus in the other lung by "+++";
virus pneumonia involving the larger part of both lungs by "++++";
death of the mouse within four days, exhibiting the typical syndrome of virus pneumonia, was assessed "++++".

The macroscopic diagnosis of virus pneumonia is relatively simple. The even dark-red discolouration of the involved parts of the lungs strikes the eye immediately. At first sight it resembles liver. It is important to dissect each mouse the same interval after infection. After 4 days the lung symptoms increase due to secondary dissemination and more animals will die. The final calculation of the 50% infection dose (I.D. 50) was made according to Reed and Munch, and "++" was assessed as 50%. In a strain well adapted to mice the I.D. 50 usually lies between 10^{-5} and 10^{-7}. Here I would like to point out two possible sources of error. In the first place, mouse pneumonia might occur. For the more experienced worker this presents no difficulties, the lungs usually being less even and less intense in colour. In the second place the virus pneumonia might be obscured by bacterial infection. The latter is clearly recognizable from the bright red colour and the foamy structure of the tissue. Mice in which these symptoms are found have to be left out of consideration, as the pattern is changed in consequence of these complications. The extent of the virus pneumonia is influenced by the bacterial inflammation and the mice often die from the complicating infection.

The determination of the egg I.D. 50 of mouse-lung suspension is

also carried out with successive 10-fold dilutions. We use three eggs for each of 6 dilutions. A haemagglutination check is carried out after two days. The eggs are put into the refrigerator for at least two hours to prevent bleeding and are harvested. Here again the calculation of the I.D. 50 according to Reed and Munch follows. Normally it lies between 10^{-6} and 10^{-8}.

The amount of antigen present in the mouse-lung suspension is determined by means of the C.F.R. We make up a series of 2-fold dilutions in the broth mixture, commencing at a 10-fold dilution. The dilution fluid is 0.9% saline.

The concentration of serum used is 12 times that required to fix complement in the presence of excess mouse-lung virus suspension. The titration is then carried out in the normal way with 2 exact units of complement, storing overnight in the refrigerator, and adding the haemolytic system the next day.

After formol fixation for at least 48 hours the lungs are embedded in paraffin wax in the usual manner. Sections 9 μ thick are cut and affixed to object glasses using a minimum of albumin. The slides are then passed through xylol and ethanol to water and hydrolysed at 60° C in 1 normal HCl for 20 minutes. After rinsing rapidly in water they are then treated with Schiff's reagent.

Preparation of Schiff's reagent:

Dissolve 1.5 gr basic fuchsin powder (Anachemia) in 200 cc boiling aqua dest.

Cool, and add 1.5 gr $K_2S_2O_5$.

Allow to dissolve, and add 3 cc concentrated HCl. Keep in the dark.

Do not use before colour has been reduced to a very light brown (12—24 hours).

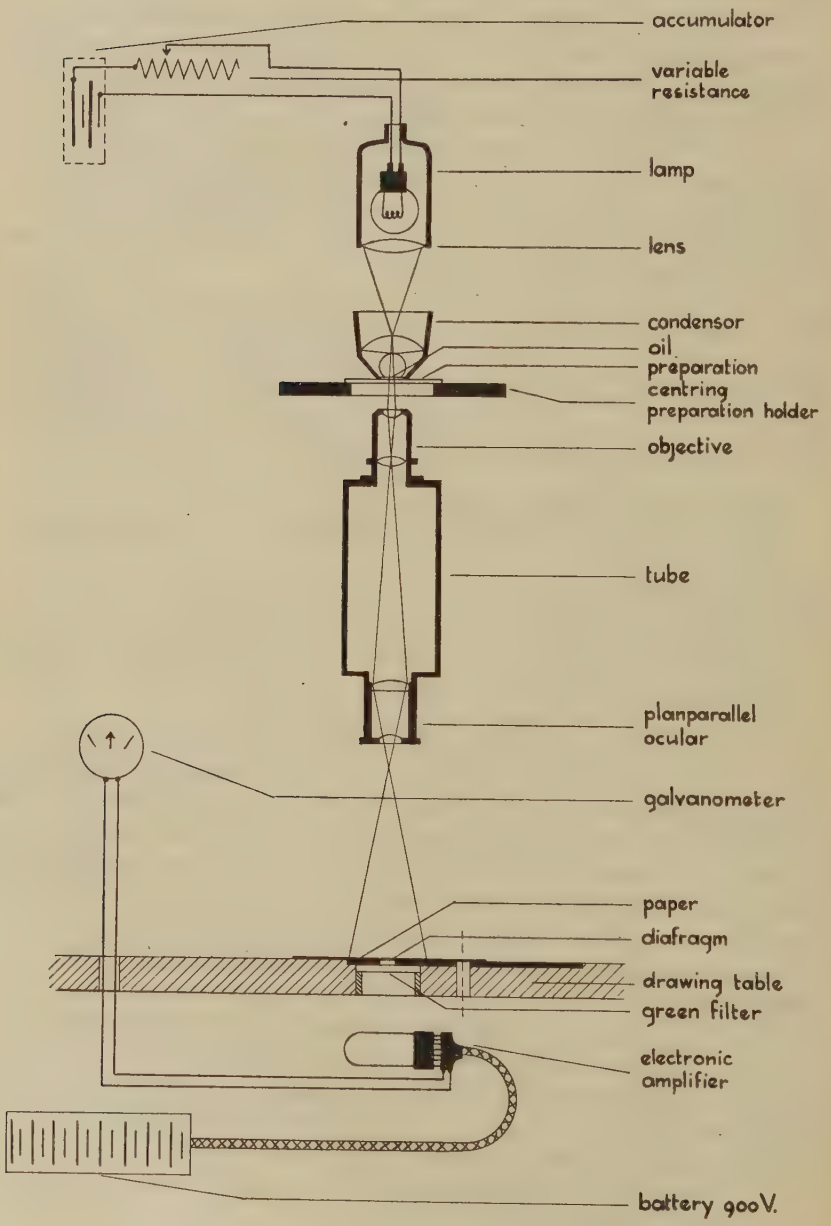
The duration of treatment with Schiff's reagent is dependent on the age of the reagent. Usually 20 minutes is sufficient. Too short a reaction time does not allow the process to come to an end and the results are not reliable. Within reason, overtreatment does not lead to impaired results. The excess reagent is neutralized by immersion in three successive baths of SO_2 water, followed by rapid rinsing with water. After dewatering, cover slips are affixed in "Cedax". (According to Lison, Harleco Synthetic Resin is better, but this was not available).

The apparatus used in our laboratory for measuring the intensity of the fuchsin-red colour in the tissues is a modification of that devised by Lison (Fig. 2). We use a Leitz micro-drawing apparatus. Objective: Zeiss apochromate 1/12; N.A. 1.2; magnification 100 $\times$ Ocular: Leitz "periplan" 10 $\times$.

A hole is drilled in the projection table and a diaphragm inserted.

We used a diaphragm of 1 mm diameter. The distance from the front of the ocular to the table was adjusted to 50 cm. Below the table diaphragm is a rotating disc containing a green filter, which

Fig. 2 Schematic representation of the micro-photometer.



can also be used as a shutter. The image of the slide is projected on to a sheet of thin white bank paper. Underneath it is a sheet of cartridge paper on which is a thick black graticule with the intersection exactly above the centre of the diaphragm. The graticule can be seen through the sheet of bank paper. Both sheets of paper are folded back during measurement.

When calibrating the apparatus we found that a series of readings for identical situations were not precisely reproducible, and that the fault appeared to be in the disc mechanism containing the filter. We therefore left the filter in a set position throughout observation on each preparation and closed the opening of the diaphragm with the cartridge paper.

Under the diaphragm and filter is an electronic amplifier of the R.C.A. 912 A type, the same amplifier as Lison uses. The anodes are fed by 10 dry batteries of 90 volts each, which can be replaced by a mains unit feeding a potential divider. The advantage of this amplifier is that it not only converts the light entering into values of electric current, but at the same time amplifies the current enormously. We kept the wiring diagram more simple than that utilized by Lison (Fig. 2).

A Pye galvanometer (Cambridge type) is directly connected to the output. This galvanometer is sensitive enough to eliminate the need for further amplification such as Lison found necessary. The room in which the apparatus is set up is maintained at a constant temperature of 22° C. The amplifier and batteries together with all wiring are kept dry by placing silica gel in the cabinets.

As a light source we use the 6-volt 30-watt lamp built into the drawing-apparatus. During adjustment this is fed by a transformer. During measuring we use a 6 volt secondary battery, such as manufactured for automobile use. Between this and the lamp we use a variable resistance to adjust the quantity of light.

Before commencing to observe, the diaphragm in the projection table is closed off, and the galvanometer adjusted to zero. This corrects for the "free-running" value of the electronic amplifier. Electronic amplifiers have the unpleasant property that, at high tensions, i.e. high sensitivities, they always pass a slight current without being exposed to light. One could take the course of measuring this current and correcting for it during the subsequent calculations, but an advantage of the galvanometer we used is that the pointer can be adjusted to zero.

After insertion of the preparation and correction for "free-running", an empty spot in the preparation is focussed (in our case for instance the lumen of a bronchus). One should certainly not observe outside the preparation as this would give rise to error. This is the "blank

observation" at which the galvanometer is adjusted to read 100 by varying the resistance of the light source circuit. After every five observations the 'zero' and '100' readings are checked and if necessary corrected. As the blank observation does not always give the same reading throughout the entire preparation (refractive error) it is necessary to execute this observation as near as possible to the cells to be observed. Notwithstanding this precaution the galvanometer indication may increase or decrease 1 or 2% in course of time. These small differences may be caused by fluctuations in the tension of the anode batteries, by peculiarities in the light source or by fluctuations in the tension of the accumulator feeding the light source.

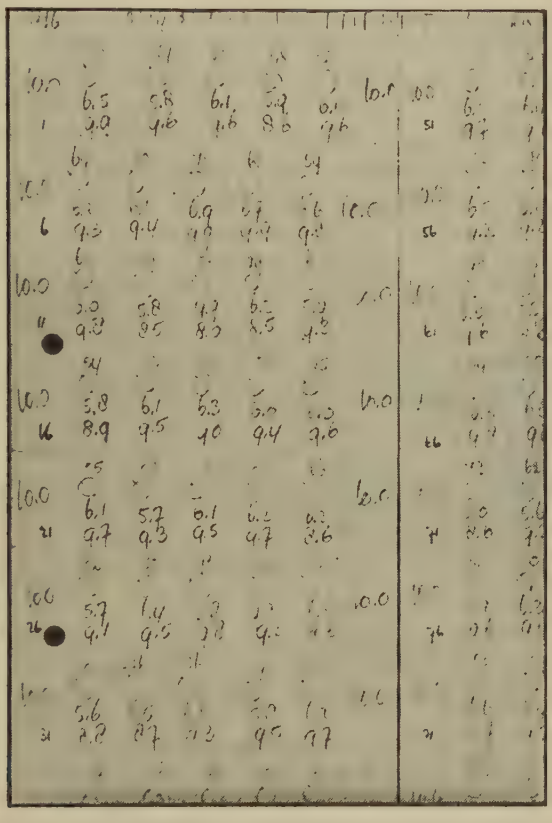
Each of these errors is slight, but together they may be enough to be observable at the galvanometer. If after five measurements the blank observation differs from the set value more than 2%, we discard the measurements of the group concerned. We correct for small fluctuations in the calculation. If repeated fluctuations in the blank observation occur we know from experience that this is usually caused by a falling-off in the tension of one of the anode batteries. (The accumulator charge is always adjusted immediately after use). The life and the tension steadiness of the anode batteries may be considerably increased by setting up the entire apparatus in a dry room.

The actual measuring takes place as follows: The nucleus of a cell to be measured is centred in the diaphragm aperture. The aperture is chosen such that its diameter is less than one third of that of the smallest nucleus to be measured. It remains so throughout the entire experiment. The outline of the nucleus is then copied on to the paper on the table. Both papers are then folded back and the galvanometer reading noted. A similar reading is then taken through the cytoplasm of the same cell, at a point at least one aperture-diameter from the nucleus. Five cells are measured in this way, and the blank observation is re-checked and if necessary re-adjusted to 100.

Further measurements were made in similar series of 5. All measurements are noted on the paper under the outline of the nuclei to which they refer. At the top of the paper the preparation containing the cells measured is noted, together with the date of measurement (Fig. 3). I found that error may creep in during the drawing of the outline of the nuclei. Each observer has his own constant subjective determination of the position of the nuclear border, which is too vague to lay down objectively. The maximum variation between observers is about the thickness of a pencil line. To minimize this effect, all measurements were done by one and the same person. The procedure is of necessity a monotonous one, and cannot be maintained accurately for more than a few hours a day. A maximum

of about 250 cells per hour can be dealt with. We have already measured over 100,000 cells, apart from the many observations necessary for calibrating the apparatus.

Fig. 3 A typical record sheet.



A number of check determinations were carried out as follows: First the blank reading was adjusted to 100. Then the reading of a nucleus or cytoplasm was noted. The light source resistance was adjusted such that the galvanometer reading was halved. The blank observation was repeated, if necessary adjusted to 50, and the percentage adjustment noted. A new measurement through a cell is made, the reading being then doubled by once more adjusting the resistance of the light source. Finally a blank observation is made once more, once more adjusted if necessary to 100, and once more the percentage adjustment noted.

The percentage deviations noted have been tabulated in groups

according to the absorption of the cell concerned (Table XVIII). It is to be clearly seen that the distribution of deviation percentages is purely random. The check demonstrates that no constant deviation is present, but that the relation between galvanometer reading and light absorption in the cell is linear.

We also executed a series of over 20,000 measurements comprising 200 each of 100 cells, and checked the galvanometer readings for reproducibility. It was found that the only factor exhibiting a correlation with the deviation values was time. When both anode batteries and light source accumulator are at the correct tensions, the deviations were restricted to 1% in 10 minutes. We have never come across deviations of more than 2% (independent of time).

TABLE XVIII

The accuracy of readings obtained with Lison's micro-absorption apparatus

absorption reading	No of readings			
	no. deviation	1% dev.	2% dev.	Total
95—100	200	—	—	200
90— 94	199	1	—	200
85— 89	197	3	—	200
80— 84	200	—	—	200
75— 79	198	1	1	200
70— 74	199	1	—	200
65— 69	198	2	—	200
60— 64	197	3	—	200
55— 59	198	1	1	200
50— 54	199	1	—	200
45— 49	199	1	—	200
40— 44	197	3	—	200
Totals	2381	17	2	2400

We encountered greater deviations than these during the measurements proper. They appear to be caused by other factors, namely error in selecting sites for blank observations. But the most convincing proof of the reproducibility of our results was yielded by a large series of measurements carried out on preparations of untreated lung tissue. At the same time these measurements demonstrate the tenability of the basic concept by yielding identical results for all untreated preparations.

The calculations.

As stated above, there is linear correlationship between the reading and the light absorption through the cell. Lison applied a formula converting the galvanometer units into an exponential series. We

commenced by applying this formula, but found that it exhibited shortcomings, particularly at low concentrations and at very high ones. The method subsequently adopted was that of setting up empirical fuchsin-concentration standard curves, by measuring several series of concentrations with the micro-absorption apparatus.

The question whether the absorptions measured were caused by D.N.A. or by other substances was disregarded, since every deviation from the normal-tissue distribution curve must have been brought about through the virus infection.

We read off conversion tables from the fuchsin standard curves, permitting absorption values to be converted to fuchsin-concentration values. Corrections were applied for permissible deviations in blank readings (less than 2%). This concludes the determination of D.N.A. concentration values for cytoplasm. It should be emphasised that all these values are purely of relative significance.

As described above, it was possible to take the volume of the nuclei into account in the nuclear calculations. The projected areas of the nuclei were measured from the outlines as drawn, by means of a planimeter. The value obtained was multiplied by the relative fuchsin-concentration value.

The relative D.N.A. concentration values for normal nuclei as converted from absorption values, are 100 to 200 times those for cytoplasm.

We attempted to establish correlation between the relative D.N.A. concentration values of the nucleus and the cytoplasm of each cell. As these calculations yielded no new conclusions, they have not been included in this paper.

Arrangement of the experiment.

A large number of mice, divided into groups of four, were given a heavy dose of potent influenza virus adapted to mice. A series of 7 dilutions (10^{-1} to 10^{-7}) of the same virus suspension was prepared and administered intranasally to 7 more groups of each 4 mice, under ether. Six groups of each 3 eggs were infected with the same virus suspension in dilutions of 10^{-4} to 10^{-9}. These last two series were intended as a check on the infection titre (I.D. 50) of the virus suspension.

Each half hour, one of the 4-mouse groups infected in the first series was chloroformed. They were then dissected, and the lungs extirpated. The left lung from each mouse was used for the determination of infection titre for mice and eggs, and of the antigen titre by means of the C.F.R. The technique was as described under "materials and methods". The right lung of each mouse was immersed in the fixation fluid. Hence we had a series of preparations and titre

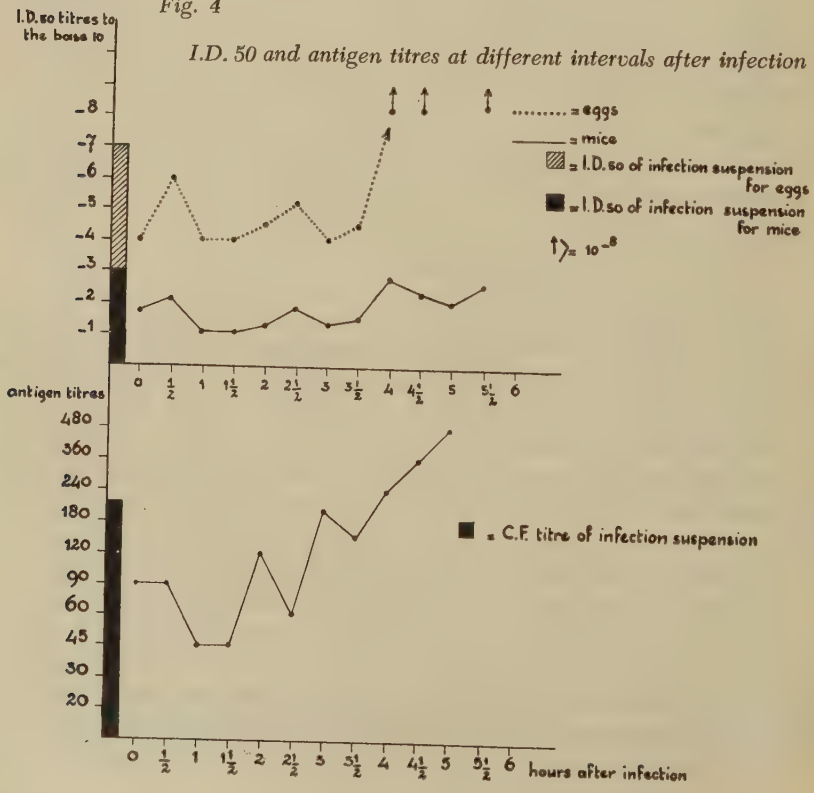
determinations for each half hour from immediately after infection up to 6 hours after infection. A number of check experiments were carried out concurrently. A similar procedure was applied to lungs from uninfected mice. In addition, mice were intranasally infected with dilution fluid, with a suspension of normal mouse-lung, and with the mouse-lung virus suspension after inactivation by heating for half an hour at 56° C.

A complete series of mice were treated with this last suspension, chloroformed and dissected also at half-hourly intervals. It proved unnecessary to treat complete series with the other check-preparations, and we confined ourselves to a number of samples.

For the sake of completeness, we may add that we also treated mice intranasally with aqua dest. Microscopic examination of a few lungs from mice chloroformed 5½ and 6 hours after treatment, disclosed inflammation symptoms, including damaged cells in the bronchial epithelium, blood vessels distended by blood, and leucocytes in the tissues. We therefore excluded this series from the check experiments.

Fig. 4

I.D. 50 and antigen titres at different intervals after infection



The results.

A considerable number of graphs were prepared from the resulting figures, most of which require no explanation, and I will therefore restrict myself to a brief discussion of them.

Figs 4, 5 and 6 represent the infection and antigen titres. The first point of note is that the I.D. 50 immediately after infection is generally lower than half an hour later. In my opinion the reason is that the suspension takes a little time to travel through the trachea to the lung. It is possible that anaesthesia also plays a part in this, though to what effect is not clear. One could imagine that the paralysis of the cilia would reduce the time required for the suspension to reach the lung, but the ether might, on the other hand, tend to reduce the virus concentration and the virus adsorption in the lung.

In every case, the antigen titre commences to rise 2 hours after infection. The striking feature is that from that moment there is no continuous rise in titre, and that on the contrary they fall off, and do not rise again until 3½ to 4 hours after infection. From then

Fig. 5 (as 4)

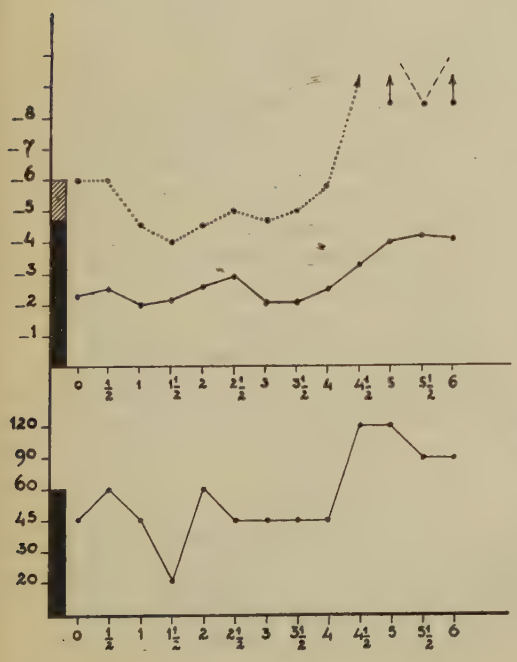
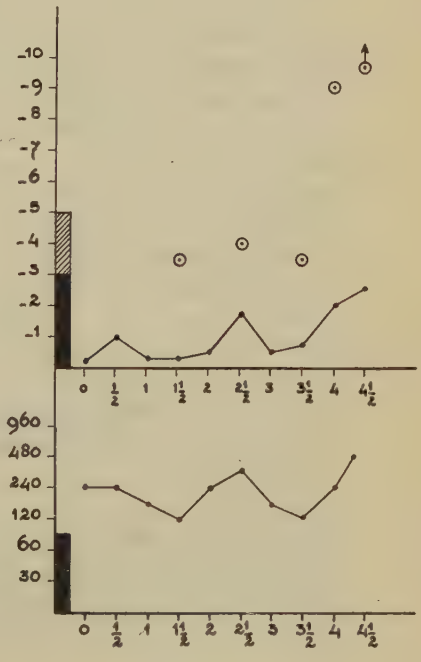


Fig. 6 (as 4)



onwards constant high titres are observed. Nowhere is there a case of clear successive titre rises.

The patterns exhibited by the course of the infection titres, both for mice and eggs, correspond in general with those of the antigen titres. Here as well the titre falls after the first half hour, to rise again 2 hours after infection. The summit is reached at about 2½ hours, followed by a fall. A second rise is observed 4 hours after infection. It is not quite clear whether the subsequent course is a further fall, or, as with the antigen titre, a gradual, continuous rise.

It goes without saying, that the utmost care has to be employed in drawing conclusions from these experiments. We hope to repeat them in the near future, but the large numbers of mice (400) and eggs (300) required are something of a handicap, to say nothing of the amount of work involved *.

The I.D. 50 for eggs is considerably higher than for mice. The influenza strains used had undergone at least 6 mouse passages, and it may be excluded that the strain was not sufficiently adapted to mice. The values of the I.D. 50 titres for eggs are all remarkably high in comparison to normal egg-adapted virus preparations. Four hours after infection, all 3 eggs inoculated with a lung-suspension dilution of 10^{-9} became positive. In the experiment recorded in Fig. 6 shortage of eggs of the required incubation period led to some of the I.D. 50 determinations being omitted. All 3 eggs inoculated in this experiment with a 10^{-10} mouse lung suspension prepared 4½ hours after infection, exhibited haemagglutination.

A possible explanation for these unusually high I.D. 50 titres may be the extremely short lapse of time (less than 5 minutes) between the chloroforming of the mice and the inoculation of the eggs. Normally this phase takes up several hours at least. Observing that there is an initial fall in titre, it is clear that the earlier the re-inoculation takes place, the higher will be the titre.

Fig. 5 shows clearly that there is no constant relationship between the I.D. 50 titres for mice and for eggs, and moreover there is none between these two respectively and the antigen titre values. The D.N.A. metabolism experiments required the first series of mice to be infected with a very heavy dose of virus. This might have influenced the titre values obtained from the first two series, but the experiment recorded in Fig. 6 where we purposely used a lighter infection dose,

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* Leaving the preparatory work out of account 4 people are required for a full 8 hours on the first day. Harvesting the eggs two days later and checking the haemagglutination ability occupies about 10 man-hours, the C.F.R. 6 man-hours, and the dissection of the mice including the checking of the lungs a further 8 man-hours.

yielded results not substantially differing from the other experiments (Figs 5 and 6).

It is no easy matter to deal with the results of the considerable number of observations on the D.N.A. content of cells, as measured by the absorption of green light after subjecting the preparations to Feulgen's reaction. Obvious changes in D.N.A. content were apparent during infection, but they are somewhat difficult to interpret. It should be stressed that check observations, whether carried out on normal mice or on mice treated with neutral material, yield identical results.

Before going on to discuss the graphs, there are a few relevant points regarding the preparations measured. We infected the mice intranasally with tremendous quantities of virus, and may assume that the major proportion duly arrived in the lungs. It will have attached itself to the susceptible cells present there, or have penetrated them. But it is doubtful whether all the susceptible cells will have been reached simultaneously, and it is indeed doubtful whether they can all be reached at all. Uninfected cells will therefore be found next to infected ones, and the infected ones will not all have been penetrated by the virus at the same moment. Different cells may therefore exhibit different stages in the virus cycle. There is no external feature of the broncho-epithelial cells in Feulgen preparations, that can establish whether they are infected or not.

The complications increase as the infection progresses. A proportion of the infected cells will be destroyed, either as a result of virus action, or because they have become too fragile to withstand the rigours of histological manipulation. These cells will not have figured in the measurements, as we took care to select only intact elements. The consequence is that the proportion of non-susceptible and uninfected cells will tend to increase. This reasoning is supported in fact. We subjected a number of the preparations to haematoxylin-eosine colouration, and were able to establish that as early as 3½—4 hours after infection, degenerative manifestations are traceable. The lumens of the bronchioles contain desquamated epithelial cells, some of which appear to be intact, but some to be seriously damaged. The superficial layers of the bronchi also exhibited damage. The pattern agrees with Straub's observations (35), though he did not encounter it so soon after infection. This may have been due to the much lower virus concentration he employed. The high concentrations we used were dictated by the requirement that as many cells as possible should be infected. If only a few cells were infected, their effect on the observation would be too small to be significant. Moreover, variation in the percentage of infected cells would not only be apparent as between preparations, but as between parts

of the same preparation as well. In both cases, the divergence of observations on infected material from those on check preparations would vary in size though not in sign. Hence, it is impossible to estimate standard deviations.

These considerations do not rule out an assessment of the accuracy of the method, for which we may make use of observations on check preparations. The cells were first grouped together according to D.N.A. content as in Table XIX. The number of observations for each group and their percentage of the total observations are noted. Further, the cumulative percentage of the total observations up to and including each group are tabulated. Standard deviations for both the groups and cumulative percentages have been calculated and included (columns 4 and 6). The same procedure has been applied to the light absorption values of the cytoplasm, and the results set out in Table XX. Once more, we feel constrained to stress that the column 1 values are merely relative.

TABLE XIX

D.N.A. content of normal broncho-epithelial nuclei, as calculated from green light absorption readings after Feulgen treatment

I	II	III	IV	V	VI
D.N.A. content	No of observations	% of total	S.D.	cumulative %	S.D.
200	3	0.5	0.33	0.5	0.33
250	13	2.2	0.62	2.7	0.69
300	63	10.7	1.27	13.4	0.40
350	187	31.7	1.91	45.1	2.04
400	183	31.6	1.90	76.1	1.75
450	74	12.5	1.37	88.6	1.31
500	37	6.3	1.01	94.9	0.91
550	23	3.9	0.81	98.8	0.47
600	4	0.7	0.37	99.5	0.33
650	2	0.3	0.29	99.8	0.24
700	0	0.0	—	99.8	0.24
750	1	0.2	0.24	100.0	0.17
	590				

We might have taken the course of expressing the results of the investigation, as tabulated in Tables XIX and XX for the check preparations, in the form of a histogram. They convey more, however, when the cumulative percentages are plotted against the absorption values (Figs 7—34). The curves from Figs 7 and 8 (normal preparations) have been superimposed in broken line on Figs 9 through 34.

In the given circumstances we considered curves deviating more than 6% from the standard curves to be significant. A deviation to left indicates that the absorption values of the majority of the nuclei or cytoplasm were lower than those for the check preparations, while a deviation to right indicates a higher average absorption.

TABLE XX

Green-light absorption readings for normal broncho-epithelial cytoplasm after Feulgen treatment

I	II	III	IV	V	VI
D.N.A. concentration (absorption)	no. of observations	% of total	S.D.	cumulative %	S.D.
0	17	2.9	0.71	2.9	0.71
1	23	3.9	0.81	6.8	1.04
2	23	3.9	0.81	10.7	1.27
3	36	6.1	0.99	16.8	1.54
4	43	7.3	1.08	24.1	1.76
5	49	8.3	1.14	32.4	1.92
6	37	6.3	1.01	38.7	2.00
7	42	7.1	1.07	45.8	2.05
8	39	6.6	1.03	52.4	2.05
9	46	7.8	1.11	60.2	2.01
10	44	7.4	1.09	67.6	1.92
11	34	5.8	0.97	73.4	1.82
12	25	4.2	0.84	77.6	1.71
13	22	3.7	0.79	81.3	1.60
14	28	4.7	0.89	86.0	1.42
15	20	3.4	0.76	89.4	1.26
16	22	3.7	0.79	93.1	1.04
17	7	1.2	0.47	94.3	0.95
18	9	1.5	0.53	95.8	0.82
19	10	1.7	0.56	97.5	0.65
20	2	0.3	0.29	97.8	0.60
21	8	1.4	0.50	99.2	0.37
22	1	0.2	0.24	99.4	0.33
23	1	0.2	0.24	99.6	0.29
24	0	—	—	99.6	0.29
25	1	0.2	0.24	99.8	0.24
26	0	—	—	99.8	0.24
27	0	—	—	99.8	0.24
28	1	0.2	0.24	100	0.17
	590				

Results of check preparations from mice treated with saline, broth mixture, and normal lung suspension are identical to the results from untreated mice. Contrary to expectation, however, absorption

readings from mice treated with inactivated lung virus suspension deviated substantially from the other check preparations. Four of the

Fig. 7

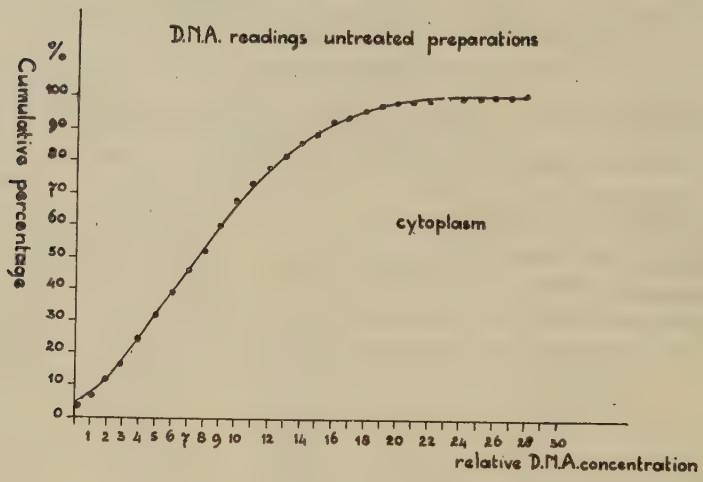
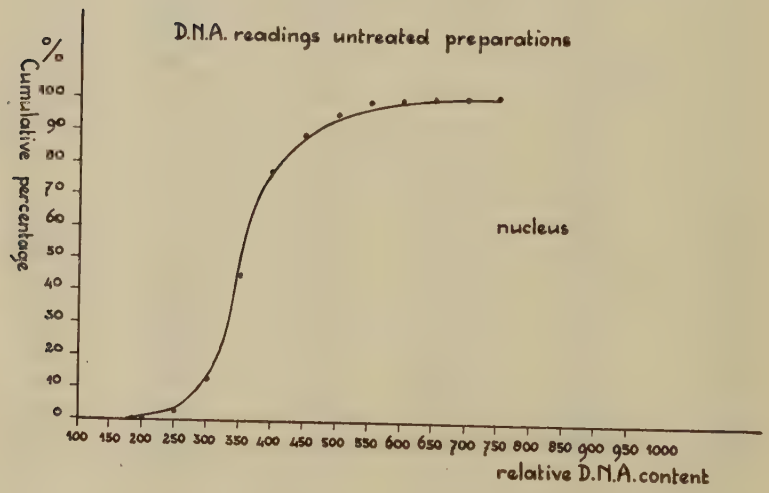


Fig. 8



curves resulting from these last measurements have been brought together in Figs 9 and 10. Up to 1½ hours from infection, the cytoplasm curves show no deviation from standard. The nucleus curves on the other hand, deviate considerably to the right as early as half an hour after treatment of the mice. In other words, the nuclei absorb substantially more green light than those in the

check preparations. The curve moves to the left 2½ hours after treatment, but remains decidedly to the right of the standard curve.

Fig. 9

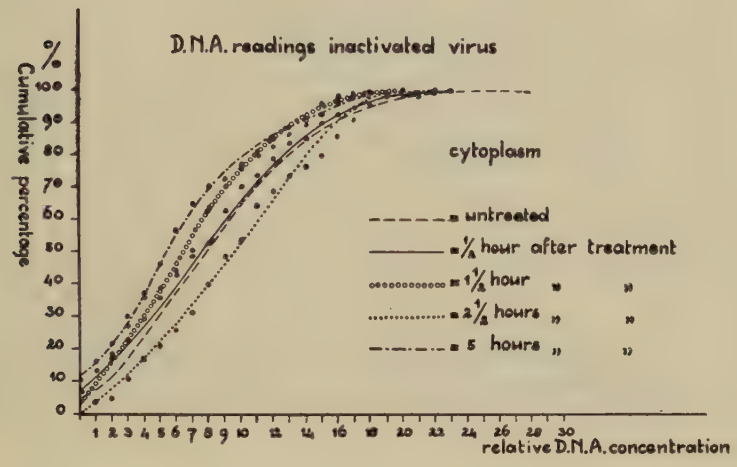
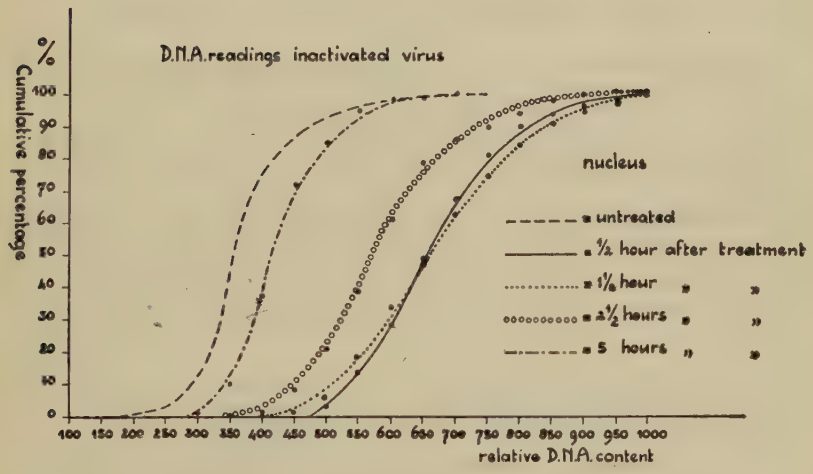


Fig. 10



At this point, the cytoplasmic absorption readings also increase. Five hours after treatment, the nuclear absorption readings approach the standard curve while those for the cytoplasm move slightly to the left. In my opinion, only one conclusion is possible — that even virus inactivated by heating at 56° C for half an hour retains its capacity to enter the cell. It attaches itself to the nucleus or

penetrates into it. After about 2½ hours, it detaches itself from the nucleus and leaves the cell. Its passage through the cytoplasm on its way out is indicated by generally higher cytoplasmic absorption readings. Perhaps it takes with it some of the nucleo-proteids contained in the cytoplasm, which would account for the lower readings recorded 5 hours after treatment.

The results of investigation with active virus include occasional divergent readings. To the reasons connected with the susceptibility of cells, as mentioned earlier in the chapter, we may now add three more. Firstly the virus suspension may contain inactive elements which nevertheless penetrate into the cells and yield high nuclear readings. Secondly, it may occur that a particle invades a cell in which the reproductive process has already commenced, and is thrown out to commence an out-of-phase infection cycle in another cell. Thirdly, active particles may invade cells not capable of sustaining a reproductive cycle, and may also subsequently commence an out-of-phase cycle elsewhere. We may further speculate on the behaviour of active particles upon invading cells already occupied by inactive virus.

Summarizing briefly, we may consider the following to be substantiated despite the theoretical disturbance just advanced:

1. Nuclear absorption readings recorded ½ and 1 hour after infection are considerably lower than standard (Figs 12 and 14).
2. Nuclear absorption readings recorded 1½, 2 and 2½ hours after infection are higher than standard (Figs 16, 18, 20).
3. Cytoplasmic absorption readings recorded up to and including 2 hours after infection are lower than standard (Figs 11, 13, 15, 17); those recorded at 2½ hours coincide with standard (Fig. 19); those recorded at 3 hours are considerably higher than standard (Fig. 21).
4. Nuclear absorption readings recorded 3 hours after infection correspond approximately to standard (Fig. 22).
5. Nuclear absorption readings recorded 3½ hours after infection are higher than standard; cytoplasmic readings are lower than standard (Fig. 23 and 24).
6. Nuclear readings recorded at 4 hours are lower than standard as regards the majority of cells measured (Fig. 26).
7. Readings recorded more than 4 hours after infection yield no clear-cut indication (Figs 27—34).

Fig. 11

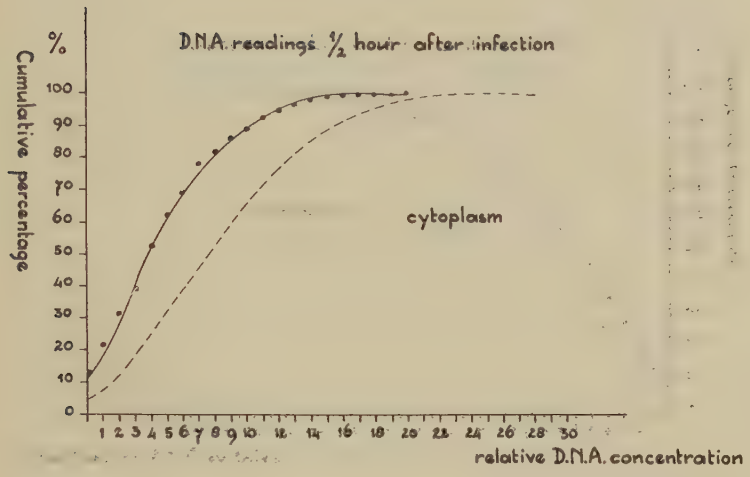

D.N.A. readings ½ hour after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 12

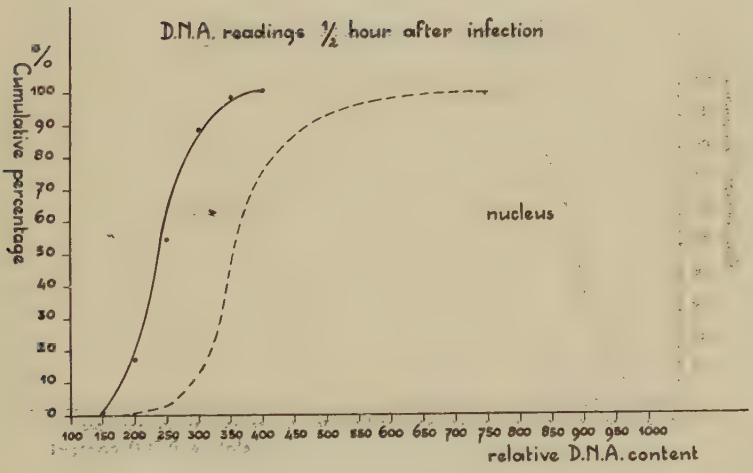

D.N.A. readings ½ hour after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 13

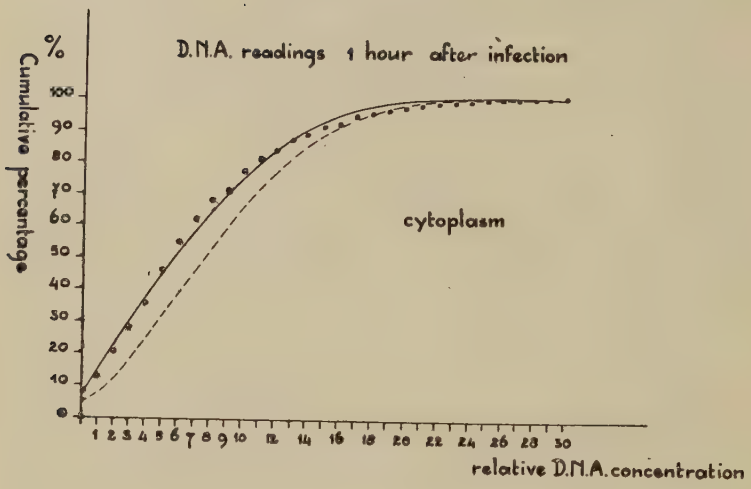

D.N.A. readings 1 hour after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 14

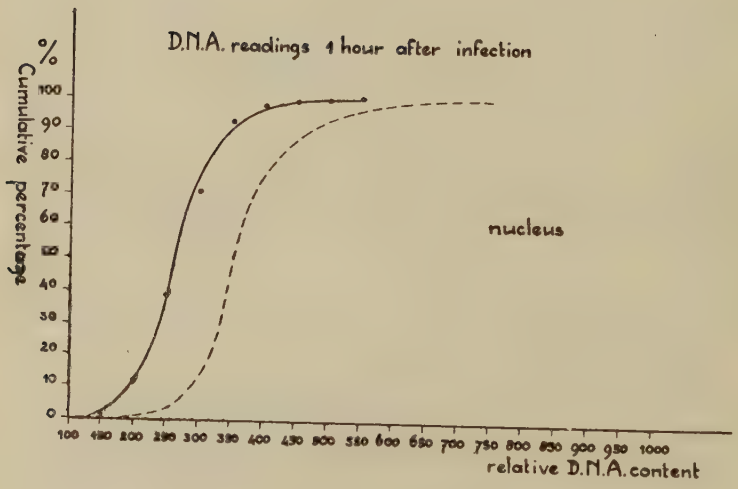

D.N.A. readings 1 hour after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 15

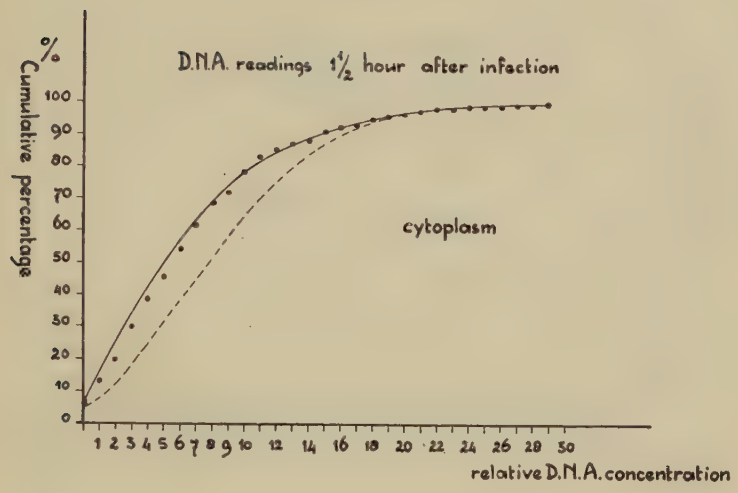

D.N.A. readings 1½ hour after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 16

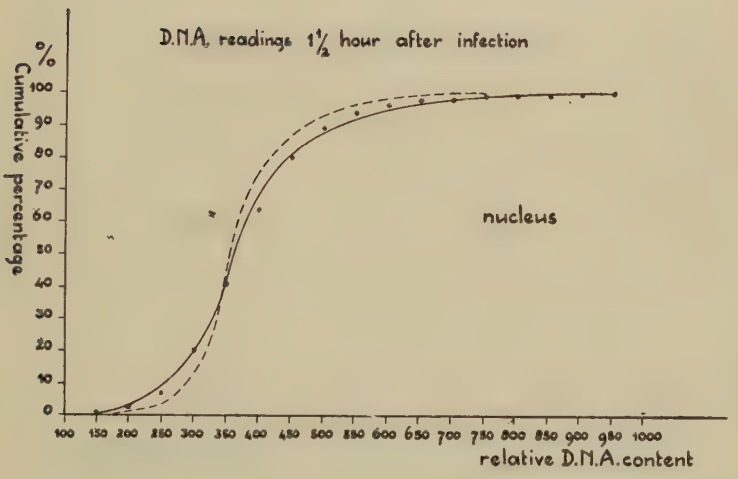

D.N.A. readings 1½ hour after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 17

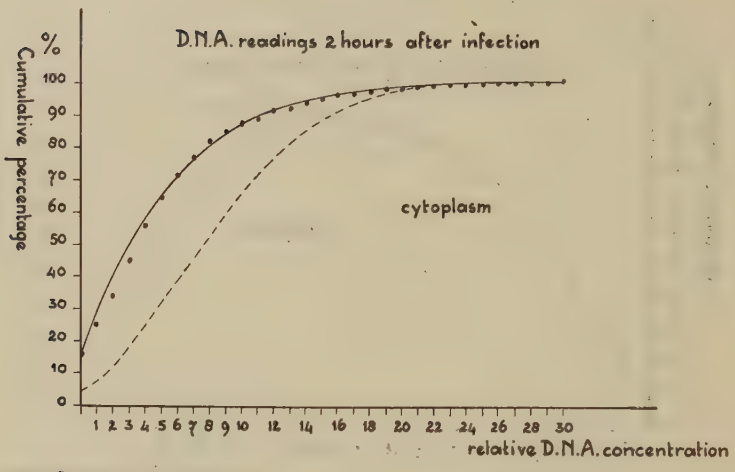

D.N.A. readings 2 hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 18

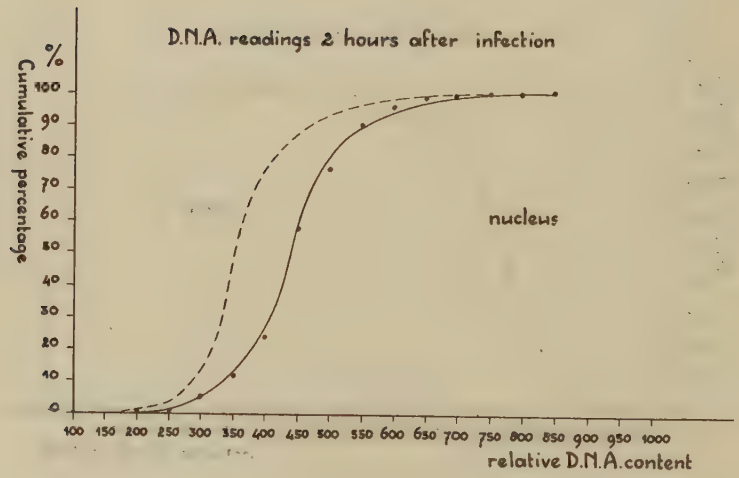

D.N.A. readings 2 hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 19

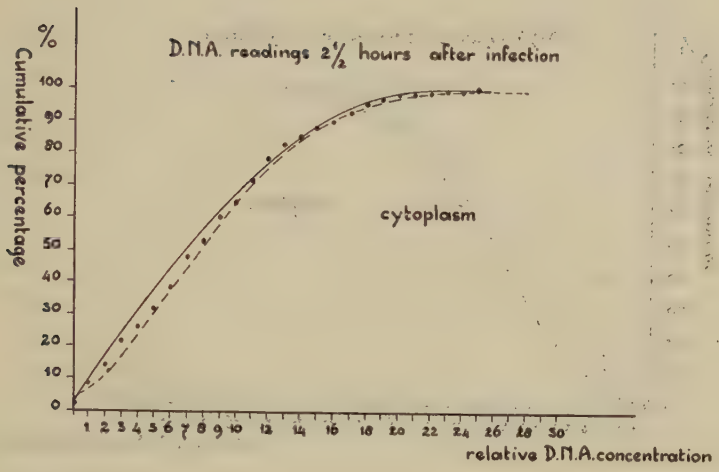

D.N.A. readings 2½ hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A.concentration

Fig. 20

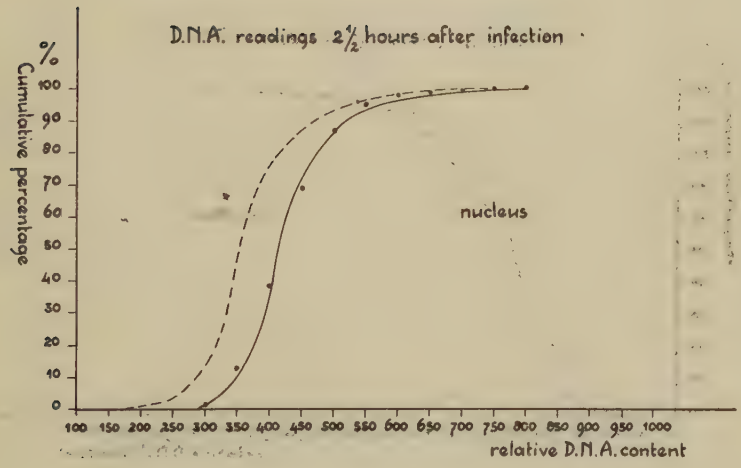

D.N.A. readings 2½ hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 21

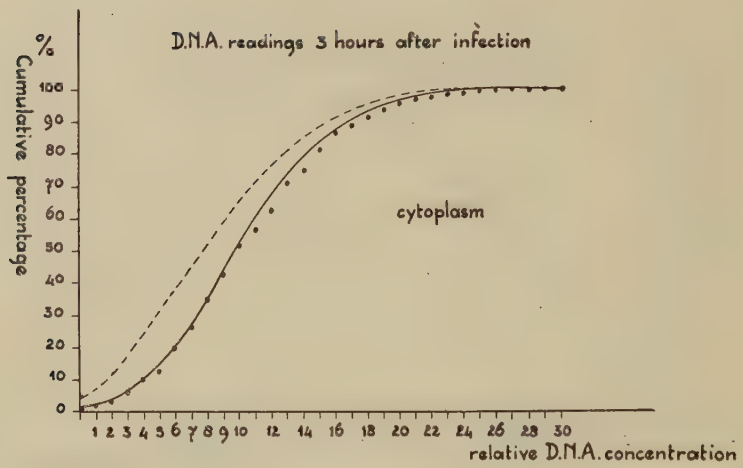

D.N.A. readings 3 hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 22

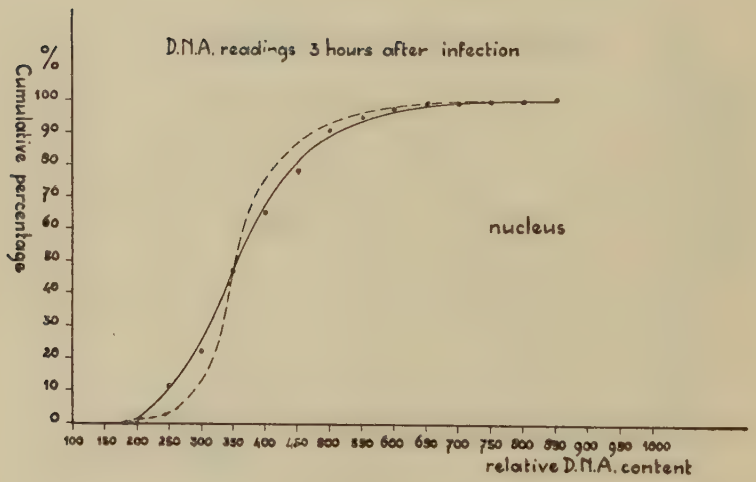

D.N.A. readings 3 hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 23

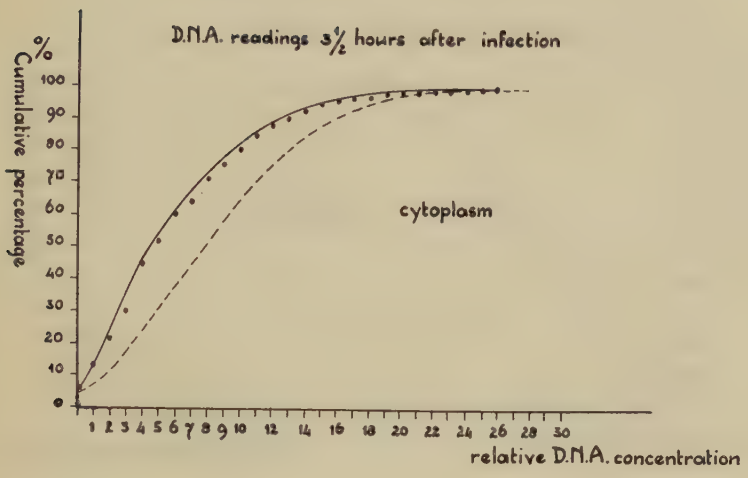

D.N.A. readings 3½ hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 24

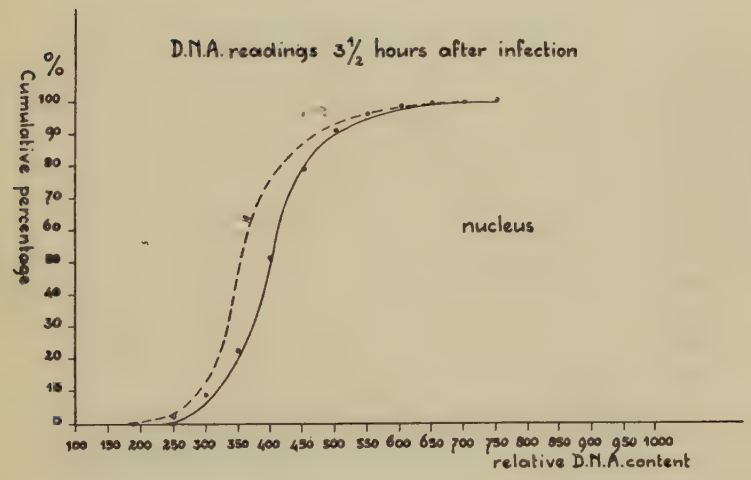

D.N.A. readings 3½ hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 25

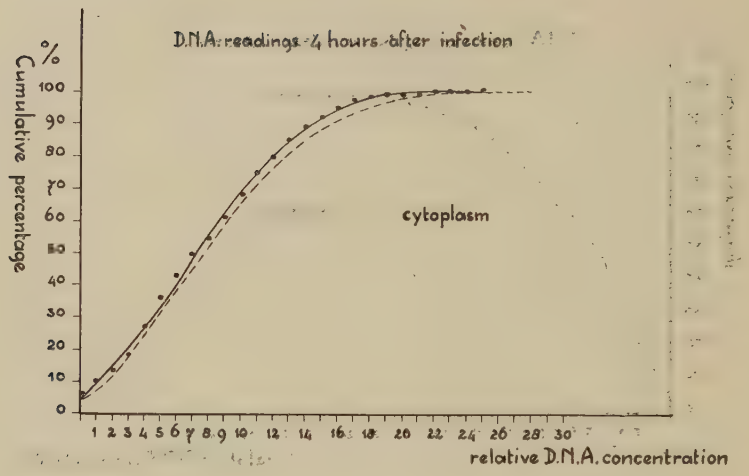

D.N.A. readings 4 hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 26

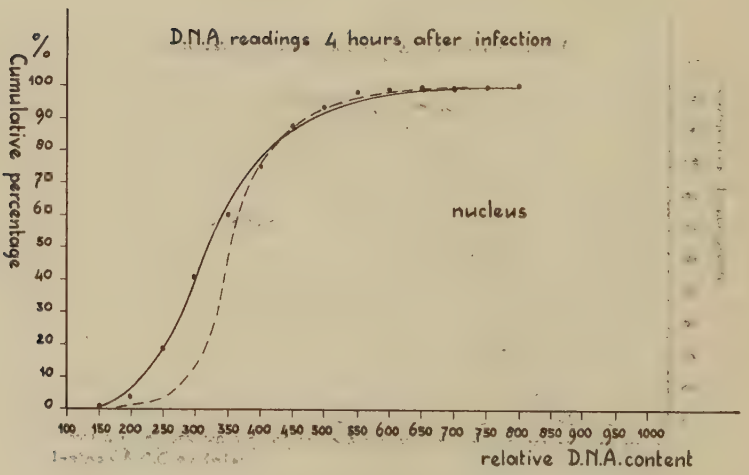

D.N.A. readings 4 hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 27

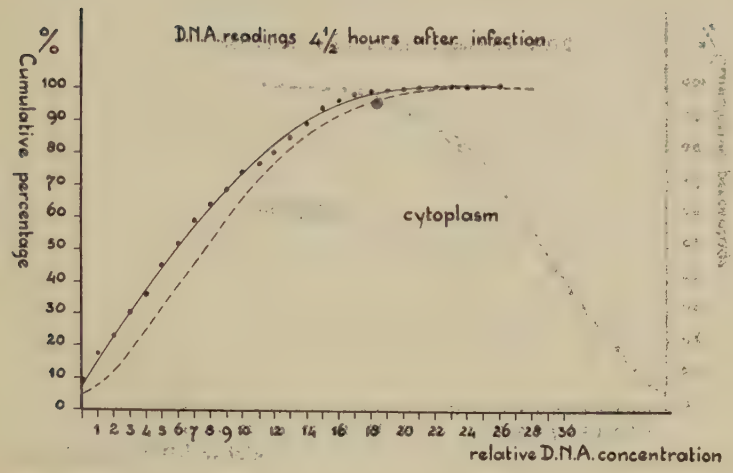

D.N.A. readings 4½ hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 28

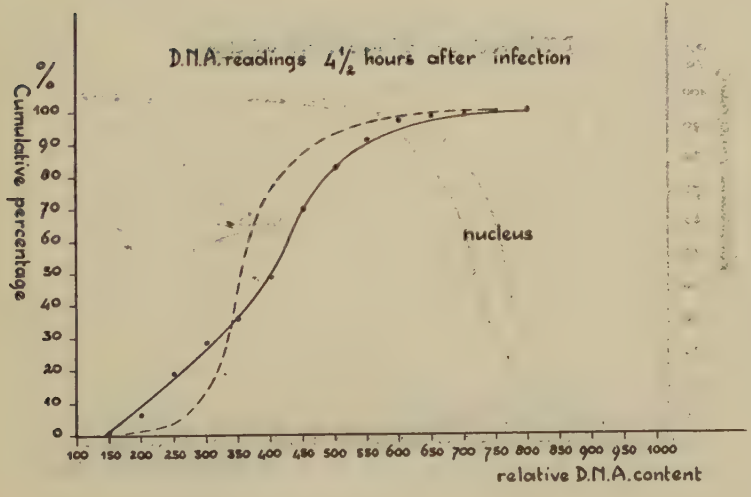

D.N.A. readings 4½ hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 29

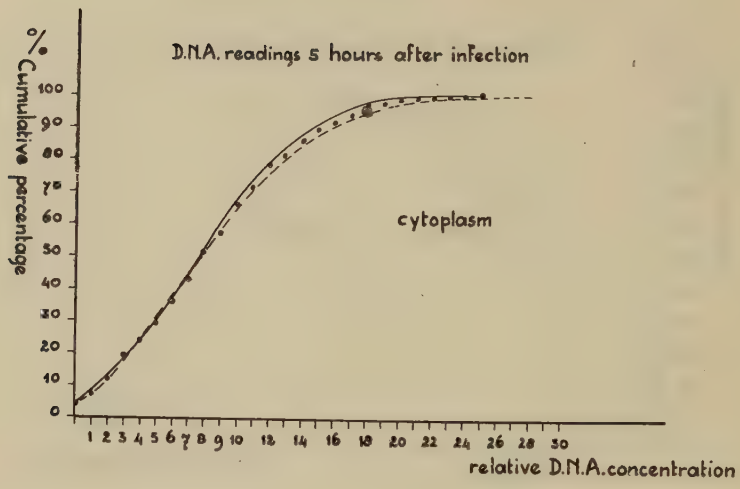

D.N.A. readings 5 hours after infection
% Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
cytoplasm
relative D.N.A. concentration

Fig. 30

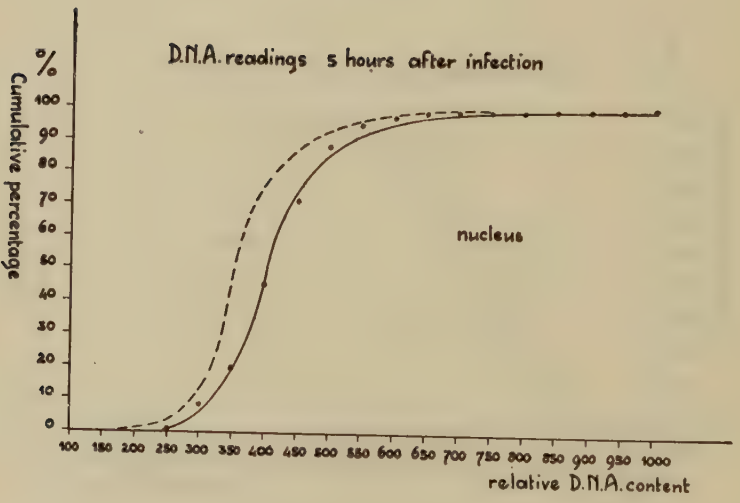

D.N.A. readings 5 hours after infection
% Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
nucleus
relative D.N.A. content

Fig. 31

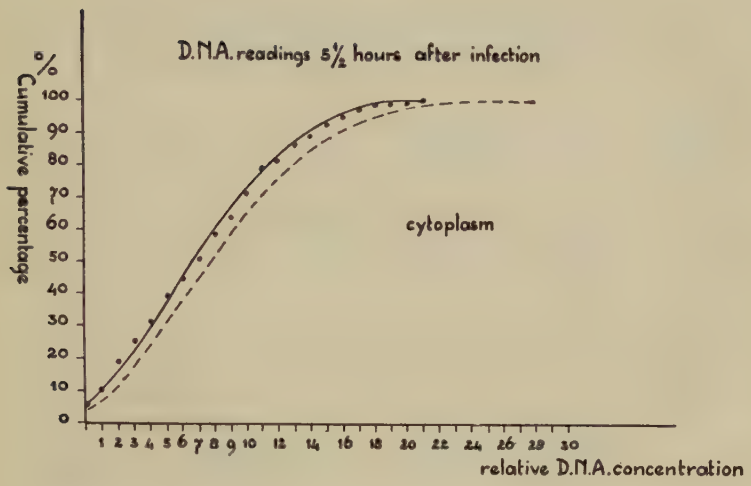

D.N.A. readings 5½ hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 32

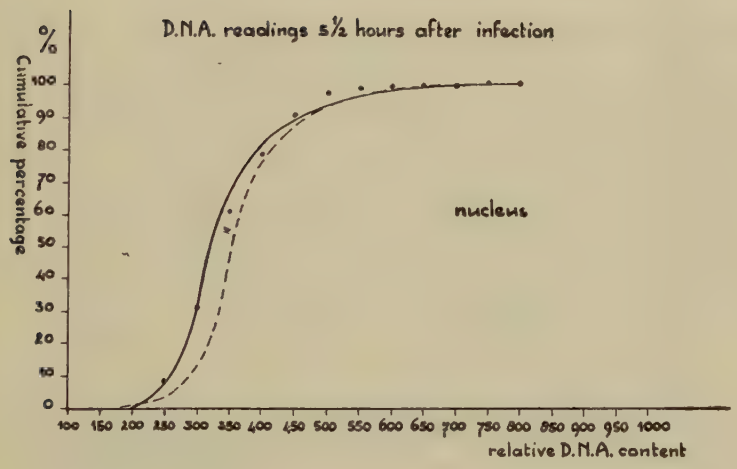

D.N.A. readings 5½ hours after infection
Cumulative percentage
%
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Fig. 33

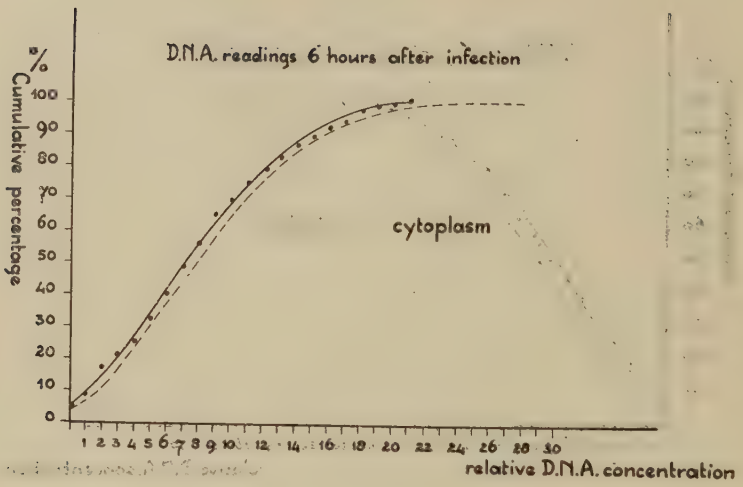
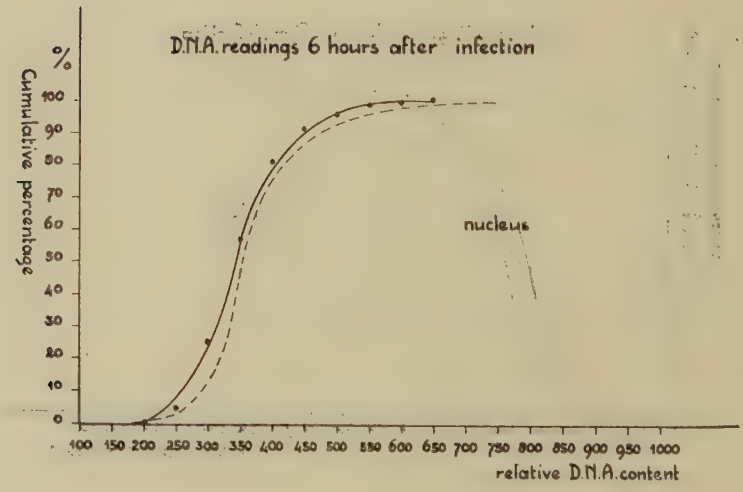

D.N.A. readings 6 hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
cytoplasm
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22 24 26 28 30
relative D.N.A. concentration

Fig. 34


D.N.A. readings 6 hours after infection
%
Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
nucleus
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
relative D.N.A. content

Conclusions.

The collation of the data we have collected about influenza virus infection in the bronchial epithelium of the mouse, permits the formation of an impression as to the course of events during the infection cycle.

1. The virus enters the cell immediately after infection. It becomes attached to the nuclear surface, and may even come to be present in the body of the nucleus itself.
2. Whilst attached to, or present within the nucleus, the virus is incapable of causing infection and even ceases to exhibit antigenic properties.
3. The total amount of D.N.A. in the nucleus and cytoplasm is less than in normal cells, ½ hour and 1 hour after infection. In other words, if D.N.A. is synthetized, the amount is less than that degraded.
4. At 1½ hours after infection the amount of D.N.A. in the nucleus exhibits an increase. This may be a result of the synthesis of virus D.N.A.
5. At 2 hours after infection there commences a discontinuous rise in antigen titre. Since a rise is also apparent with the inactivated virus, this is probably a result of:
   a) Inactive elements in the virus suspension.
   and/or b) Active elements incapable of reproduction in the cell concerned.
   and/or c) The cell concerned being incapable of sustaining the reproductive process.
6. At 2 hours after infection there is also a discontinuous rise in I.D. 50 titre. This is probably initiated by 5 b) and/or 5 c) above.
7. At 2½—3 hours after infection, the cytoplasm exhibits the presence of increased quantities of D.N.A. This may be interpreted as being caused by the same phenomena as give rise to 5 and 6 above.
8. At 4 hours after infection a continuous rise in I.D. 50 and antigen titres has commenced. At the same time lower nuclear D.N.A. readings are observed. We may conclude that this is due to the conclusion of the infection cycle and the commencement of a new one.
9. At 4½ hours after infection the percentage of cells with low nuclear D.N.A. readings and that for cells with higher nuclear D.N.A. readings have increased at the cost of that for cells in the average zone (Fig. 28). This is an indication that the second cycle is likely to proceed more rapidly than the first.

The general picture of this reproduction cycle coincides largely with Hoyle's results. There was only one point where it appeared at

first that there would be a divergence. We found increases in antigen and infection titre as early as 2—2½ hours after infection. The probable explanation is that virus particles having no part in the reproduction process leave the cells during this period.

What is new in these results is that we have a strong indication as to the site at which the reproduction process takes place. This we now consider to be either at the surface of the nucleus or within the nucleus itself.

At this stage the same problem arises as in bacteriophage multiplication, i.e. whether the virus disintegrates into its components parts, one of which (D.N.A.) induces the production of new virus components and their composition into new virus particles, or whether the virus particle remains intact, reproducing itself by a simple process of division and therefore by geometric progression.

Contrary to what has been assumed up to now, it appears that virus inactivated by heating at 56° C for half an hour retains its capacity to enter the cell, despite the loss of certain of its enzymes.

The investigation as a whole has provided us with a number of pointers as to how various types of virales develop in the cell. Apart from D.N.A. investigation we have already applied the method discussed to the measuring of other substances of importance in virus multiplication, for instance the concentration of mucoproteids in the cell.

A number of shortcomings have proved to be inherent in the method applied. For example it does not achieve the simultaneous infection of all susceptible cells, and the half-hour interval dictated by the required manipulations is really too long to provide a continuous picture. We hope to surmount these obstacles by working with tissue cultures. As regards the morphology of virus multiplication, the electron microscope will be of value, though present-day observations with this instrument have to be regarded with circumspection as the preparation of material for observation is drastic. The method of shadow casting, aimed primarily at rendering the external structure visible, is not yet satisfactory as regards the interior of the cell. It may be that impregnation with heavy metal salts such as platinum chloride, or fixation in osmic acid, may improve matters in this respect.

The methods of antigen and I.D. 50 titre determination may yet prove to yield valuable data of assistance in distinguishing between the various influenza virus strains. But apart from quantitative differences, there are also qualitative differences to be revealed, and both are of importance for the investigation of the virulence problem. It may be that the micro-spectro-photometer will be a valuable tool in this respect.

CHAPTER VI

Summary and Discussion.

In the introduction I touched upon several points of importance for epidemiological research into virus diseases. In the first place there is the demonstration and, if possible, isolation of the infectious agent, and in the second place the comparison of different virus strains with each other. Strains may exhibit differences in serological and in biological behaviour. A special problem is the study of the various factors involved in the virulence of virus strains. I have expressed these factors in a formula from which it becomes clear that for the determination of virulence one has to know the number of virus particles in the specimen, that are capable of causing infection.

The second chapter contains a description of my search for new methods of demonstrating influenza virus. When adapted to eggs, influenza virus is capable of agglutinating chicken's erythrocytes. Many investigators have preceded me in making use of this property to attempt to demonstrate virus in human material. Neither they nor I have succeeded.

For several virales the Complement Fixation Reaction (C.F.R.) was used, examining the antigen instead of the serum. This Complement Fixation Reaction for demonstrating Antigen (C.F.R.A.) is already applied for the diagnosis of variola and foot-and-mouth disease. Our efforts to demonstrate influenza virus by this method were not attended with success. The number of false positive reactions obtained was quite considerable, while in a large percentage of cases false-negative results were also observed. We are forced to the conclusion that the C.F.R.A. is of no value for influenza research.

We therefore turned to an indirect method. Firstly we added specific antiserum to the specimen containing the virus as collected from human individuals or ferrets. We then determined the haemagglutination inhibition titre of the mixture. "If this material contains virus" we reasoned "then it will subject the antibodies in the serum to fixation". In that case there will be fewer antibodies for the HaemAgglutination Inhibition Test (A.I.T.), to be executed at a later stage. We gave this method the name of "Specific Antibodies Fixation", or S.A.F. In principle it proves to be possible to demonstrate virus by it. However, difficulties arose when we subjected the sensitivity of the reaction to further examination. The

frequency with which we obtained negative results, despite the presence of virus in the garglings and nasal washings examined, turned out to be considerable. Hence we adjudged the reaction to be of low sensitiveness. The frequency of false positive results was fairly low. We came to the conclusion that despite its lack of sensitiveness, the method is reliable insofar as virus *is* demonstrated. For the purpose we had envisaged, this method was of no more use than the C.F.R.A., since we had sought a kind of "screening test", perhaps yielding too frequent positive results, but only seldom failing to demonstrate virus when present. We hope to continue our investigation and to obtain better results eventually. In itself the S.A.F. may nevertheless be of some use. Where there is no apparatus or material for isolating the influenza virus, the latter may be demonstrated by the S.A.F., though the antisera and virus for the A.I.T. will have to be obtained from elsewhere. An advantage of the S.A.F. is that the results are obtained within 24 hours.

To be in a position to execute these tests we had to find a medium in which the virus present in the garglings or nose washings was not destroyed too quickly. A mixture of bacteriological broth and saline to which 400 units of penicillin and 0.25 mgr per cc of streptomycin had been added came up to our requirements.

We developed a new micromethod for measuring influenza antibody titres, using plasma instead of serum, and the patient's own red cells instead of chicken's or human (O) erythrocytes. This method proved to be a simple and reliable test for the purpose.

We were able to study the course of an influenza epidemic in Endegeest Asylum during the winter 1948/9 and have reported the results in the third chapter. We collected specimens of blood from about 300 persons at the beginning of the influenza season (November), and again at the end of the season in April. In addition we collected serum from 89 patients who suffered from influenza during the investigation, both at the onset of the illness and ten to fourteen days afterwards. Thus we had at our disposal more than 700 specimens of serum, which we processed in various ways.

As a result of this work we were able to compare the various methods we applied. It was established that for the confirmation of the clinical diagnosis of influenza, the C.F.R. gives the best results. However this test is not able to determine which particular kind of influenza strain is causing the infection, although, of course, the group can be established. The A.I.T. lends itself better to strain determination, but it has a drawback for clinical examination in that its greater specificity ignores cases of influenza caused by strains of deviating antigenic composition. This property may be turned

to advantage in studying the epidemiology of an explosive influenza outbreak.

In studying the C.F.R. titre values we noted the fact that the acute-phase sera of patients with clinical symptoms showed extremely low titres. On the other hand the earliest specimens from individuals without influenza symptoms, which nevertheless demonstrated significant titre rises, exhibited high C.F.R. titres. These data are of particular value for investigations into the efficacy of influenza vaccination, since it is clear that the presence of antibodies, as demonstrated by C.F.R., prevents outbreak of the illness.

During the investigation there occurred 89 cases of influenza. The first of them was diagnosed at the end of December, and the last at the end of February. The problem here was whether we had to do with one single epidemic or repeated introduction of the virus into the community. To our regret we cannot provide the answer. The question is important, the more so as we isolated two different kinds of influenza strains. Both strains belonged to the A′ type. The one reacted quite well with its own and with other antisera, and the other only to low titres. Of late much attention has been paid to this point, the general opinion being that the strains reacting well (the "Liverpool" type) are more virulent than those reacting weakly (the "Stockholm" type). If we were certain that the types could not be converted the one into the other, we would have the answer to our question — the influenza virus must have been introduced into the community more than once. An argument for this may be that significant increases in the number of persons reporting sick were noted on three successive Mondays, thus directly after the weekend visits. In addition to this another argument may be that we not only diagnosed influenza in one patient twice, but also isolated virus both times. The first was of the "Stockholm" type and the second of the "Liverpool" type. If we knew with certainty that virus could not have been introduced into the community more than once, proof would have been furnished that the one type could be converted into the other.

In an effort to shed further light on the matter, we tried to ascertain whether there are any clear differences in antigenic composition between the two types. This question as well had to remain unanswered. In this connection it is interesting to note that each animal passage may bring about a change in the antigenic composition of a virus. Small differences, only detectable with sensitive methods (A.I.T.), are not important to the question in point.

All these problems regarding virulence and antigenic composition raise the need for more knowledge about the behaviour of the virus

in the cell of its host. This led us to set up a quantitative investigation in this direction. As subject we chose the metabolism of desoxyribose-nucleic acid (D.N.A.), as this substance (or polymers of it) must be considered an essential element of each virus. We were able to establish that, beginning half an hour or less after the infection, the D.N.A. content of the cell undergoes modification. We found less D.N.A. both in the nucleus and the cytoplasm half an hour after infection. Even with virus inactivated by heating for half an hour at 56° C we were able to prove that the virus either becomes attached to the nucleus or actually enters it, by recording a rise in the D.N.A. content of the nucleus. The difference between inactivated virus and untreated virus is remarkable, since the latter occasions a decrease in the D.N.A. content of the nucleus. It is important in itself, that virus inactivated by heating for half an hour at 56° C enters the cell at all.

Apart from this investigation into the D.N.A. metabolism in the broncho-epithelial cells of the mouse lung after infection with influenza virus (W.S.), we also followed the course of the infection in the mouse lung step by step by determination of infection titre (I.D. 50) and antigen titre. In broad outline we found a confirmation of the results of Hoyle, who carried out a similar investigation with eggs. But contrary to Hoyle's findings, we recorded increases in both I.D. 50 and antigen titre as early as 2—2½ hours after infection. It would seem feasible at first sight, to explain this initial rise by assuming that the infection cycle is not 4 hours, as Hoyle suggests, but only 2 hours, but investigation of the D.N.A. content of the cell rendered this unlikely. Firstly, because the D.N.A. content of the nuclei of broncho-epithelial cells is reduced in comparison to standards immediately after infection, and this is followed by a second fall only after 4 hours. Secondly because the nuclear D.N.A. content rises with respect to standards 1½ hours after infection, and a further rise is not observed before 3—3½ hours later. Thirdly, because inactivated virus is also observed to detach itself from the cell 2—2½ hours after infection. Regarding this last it is probable that virus still capable of reproduction but for one reason or another hindered in the process behaves in the same manner as inactivated virus and leaves the cell. We may well imagine this virus penetrating into cells already involved in a reproductive process or not capable of supporting reproduction, or perhaps even already occupied by inactive virus.

After the conclusion of the infection cycle (4 hours) enormous quantities of virus are released. The I.D. 50 titre of the suspension exhibits at times a rise to values even above 10^{-10} for eggs. This is also a high value as compared to the I.D. 50 titre for mice. We have

never encountered such high values, either with mouse lung suspension or even allantoic fluid. We are convinced that the reason for this must be sought in the fact that the inoculations were carried out within 5 minutes of chloroforming the mice. Other investigations have shown that the I.D. 50 titre falls rapidly during the first few hours after preparation of the suspension. This last point leads directly to the assumption that the suspensions contain in fact a high proportion of inactive virus, and therefore that our inoculations had been carried out with a mixture containing a good deal of inactive virus and a relatively small quantity of reproductive virus.

Similarly to the development of T bacteriophages in Escherichia coli, we can recognize successive phases in the development of influenza virus in the bronchial epithelium of the mouse. During the first phase, the virus enters the cell and becomes attached to the nucleus, perhaps penetrating it. Whether it loses certain of its constituent substances at this stage, as does the bacteriophage, cannot be established with certainty. There are indications that it may do so.

The virus loses its capacity to infect other cells for a certain time, even when it takes no part in the reproductive process. But this property also permits of other explanations. The D.N.A. content of the nucleus decreases during the second phase and is synthetized during the succeeding third phase, though this time probably virus D.N.A. After 2½—3 hours, a fourth phase commences, during which we observe an increase in the cytoplasmic D.N.A. concentration. Possibly this D.N.A. is virus that has not taken part in the reproduction process and is on its way out of the cell after becoming detached from the nucleus.

Finally, the "adult" virus is formed during the last phase. The entire cycle is carried through within a space of less than 4 hours. After 4 hours, the first cycle has been completed and the second has commenced.

Comparison between the results of this investigation on W.S. virus and similar investigations on other strains may bring to light data of importance to the virulence problem. In any case it would enable a number of properties of the influenza virus to be studied further. Moreover, one might observe other substances besides D.N.A. during the course of the infection cycle. In conclusion, one might apply these same methods to other virales.

Bespreking van de resultaten en samenvatting

In de inleiding heb ik verschillende punten aangeroerd, die voor het epidemiologisch onderzoek van virusziekten van belang zijn. In de eerste plaats komt het aantonen en eventueel isoleren van de smetstof. Daarop volgt het vergelijken van virusstammen. Het is mogelijk verschillen in serologisch en in biologisch gedrag te vinden. Een apart probleem vormt het onderzoek naar de verschillende factoren die de virulentie van de virusstam bepalen. Ik heb het verband tussen deze factoren in een formule samengebracht. Uit de formule valt duidelijk af te lezen, dat men dán alleen de virulentie van een stam kan bepalen, als men het aantal infectieuse virusdeeltjes kan vaststellen.

In het tweede hoofdstuk werd beschreven hoe ik naar nieuwe methodes gezocht heb, waarmede influenza virus aangetoond zou kunnen worden. Op eieren voortgekweekt influenza virus is in staat rode bloedcellen van kippen te doen samenklonteren. Van deze eigenschap hebben reeds velen vóór mij getracht gebruik te maken voor het aantonen van virus in patientenmateriaal. Mij is dit evenmin gelukt als mijn vele voorgangers.

Voor verschillende virussoorten is reeds gebruik gemaakt van de complementbindingsreactie, waarmede dan niet het serum, doch het antigeen onderzocht wordt. Deze „complementbindingsreactie voor het aantonen van antigeen" (C.B.R.A.) is reeds van toepassing voor de pokken- en mond- en klauwzeer-diagnostiek. Met behulp van deze methode is het ons niet gelukt influenzavirus op betrouwbare wijze aan te tonen. Het aantal malen, dat ten onrechte een positieve uitkomst verkregen werd, is vrij aanzienlijk, terwijl ook in een groot aantal gevallen „foute" negatieve resultaten genoteerd werden. Wij moeten tot de conclusie komen, dat deze complementbindingsreactie voor het aantonen van antigeen zonder betekenis is voor het influenza onderzoek.

Daarom hebben wij een indirecte weg ingeslagen. In de eerste plaats hebben wij het van mensen (of fretten) afkomstige virushoudende materiaal samengebracht met specifiek antiserum. Wij hebben daarna van dit serum-virusmengsel de haemagglutinatie inhibitie-titer bepaald. Indien dit materiaal virus bevat, zo hebben wij geredeneerd, dan moet dit antilichamen uit het serum binden. In dat geval zullen minder antilichamen over blijven voor de later uit te voeren haemagglutinatie inhibitie test. We hebben deze reactie

de Specifieke Antilichamen Binding genoemd (S.A.B.). In principe bleek het mogelijk op deze manier virus aan te tonen. De moeilijkheden kwamen echter pas, toen wij de gevoeligheid van deze reactie aan een nader onderzoek onderwierpen. Het aantal malen, dat wij een negatieve uitkomst verkregen, terwijl het neus- of keelspoelsel toch virus bevatte, bleek groot te zijn. De reactie is dus vrij ongevoelig. Het aantal keren, dat de proef ten onrechte een positief resultaat opleverde was vrij gering. Wij komen dus tot de conclusie, dat de methode vrij ongevoelig, maar, voor zover virus aangetoond wordt, betrouwbaar is. Voor het doel, dat wij ons gesteld hadden was deze manier van het aantonen van virus evenmin bruikbaar als de complementbindingsreactie voor het aantonen van antigeen. Wij zochten immers naar een soort „screeningstest" waarmede misschien wel te veel positieve uitkomsten verkregen worden, doch waarmede slechts een onbetekenend aantal keren geen virus aangetoond wordt, als dit aanwezig is. Wij hopen onze proeven voort te zetten en door wijzigingen in de uitvoering aan te brengen, tenslotte betere uitkomsten te verkrijgen. Op zichzelf is de S.A.B. misschien toch van belang. Daar, waar men niet over apparatuur en materiaal beschikt voor het isoleren van influenza virus kan men met behulp van deze „Specifieke Antilichamen Binding" toch virus aantonen (mits men de antisera en het virus voor de agglutinatie remmingsreactie van elders betrekt). Bovendien is het resultaat binnen 24 uur bekend.

Ten einde de bovengenoemde onderzoekingen uit te kunnen voeren was het noodzakelijk een medium te vinden waarin het in keelspoelvocht of neusspoelsel aanwezige virus niet snel te gronde ging. Een mengsel van bacteriologische bouillon en zoutsolutie waaraan per ccm 400 eenheden penicilline en 0.25 mgr streptomycine waren toegevoegd voldeed aan onze eisen.

Wij ontwikkelden een nieuwe micromethode waarmede het mogelijk is een indruk te krijgen over de hoeveelheid antilichamen voor influenza. Wij gebruikten te dien einde citraatbloed, waarvan het plasma in de plaats van menselijk serum kwam en de eigen bloedlichaampjes de toe te voegen kippen- of menselijke O erythrocyten vervingen. De methode is snel, eenvoudig en betrouwbaar.

Wij zijn in staat geweest het verloop van een influenza epidemie in het gesticht Endegeest in de winter 1948—1949 te bestuderen. De resultaten van deze studie hebben wij in het derde hoofdstuk vermeld. Bij ongeveer driehonderd personen konden wij aan het begin van het influenza seizoen (November) bloed afnemen. Aan het einde van het seizoen werd in April bij deze patienten nogmaals bloed afgenomen. Bovendien hebben wij van 89 patienten die

tijdens ons onderzoek aan influenza leden bij het begin van hun ziekte en nog eens tien tot veertien dagen later serum gewonnen. Wij beschikten zodoende over meer dan 700 sera, die wij op verschillende manieren bewerkt hebben.

Als resultaat van dit onderzoek hebben wij een vergelijking kunnen maken tussen de verschillende methodes, die wij toegepast hebben. Vast kwam te staan, dat voor de bevestiging van de klinische diagnose „influenza" de complementbindingsreactie de beste resultaten geeft. Met behulp van deze reactie is het niet mogelijk vast te stellen met welke soort influenza stam men te maken heeft, hoewel natuurlijk wel de groep bepaald kan worden. Voor de stambepaling leent de haemagglutinatie inhibitie test zich beter. Een bezwaar voor het klinisch onderzoek is echter, dat door de grote specificiteit van deze methode, influenza gevallen, veroorzaakt door stammen van afwijkend antigene samenstelling, gemist worden. Dit nadeel kan tot voordeel worden bij de bestudering van de epidemiologie van een influenza explosie.

De Complementbindingsreactie titers van de eerste sera van influenza patienten waren vrijwel zonder uitzondering zeer laag. Die van personen zonder klinische verschijnselen, maar waarbij significante titerstijgingen een infectie aanduidden, waren duidelijk hoger. Dit is van belang voor het influenza-vaccinatie probleem, omdat men de gevolgtrekking mag maken, dat het demonstreren van complementbindende antilichamen immuniteit voor influenza inhoudt.

Tijdens ons onderzoek traden 89 gevallen van influenza op. Het eerste geval werd eind December geconstateerd; het laatste geval eind Februari. Hebben wij hier te maken gehad met één epidemie, of is het virus telkens weer opnieuw in de gemeenschap binnen gedrongen? Tot onze spijt kunnen wij hier geen antwoord op geven. De vraag is temeer van belang, omdat wij tijdens ons onderzoek twee verschillende soorten influenza stammen isoleerden. Beide soorten stammen behoren tot het A'-type. De ene soort reageert goed met zijn eigen én met andere antisera, de andere groep stammen doet dat slecht. Aan dit verschil is in de laatste tijd veel aandacht besteed. Men meent namelijk, dat de goed reagerende stammen (van het type „Liverpool") virulenter zouden zijn dan de slecht reagerende (van het type „Stockholm"). Wisten wij enerzijds dat de types niet in elkaar over kunnen gaan, dan zouden wij een antwoord op onze vraag hebben: het influenza virus moet dan meer dan een keer in onze gemeenschap binnen gedrongen zijn. Hiervoor zou kunnen pleiten, dat wij bij één patient niet alleen twee keer influenza konden constateren, maar ook twee keer virus isoleerden en wel de

ene keer van het type „Stockholm" en de andere keer van het type „Liverpool". Bovendien werden op drie opéénvolgende Maandagen in aansluiting op het bezoek gedurende het week-end een beduidend groter aantal influenza gevallen geconstateerd.

Wisten wij anderzijds met zekerheid, dat niet meer dan één keer virus in onze gemeenschap kon zijn binnengedrongen, dan ware het bewijs geleverd, dat het ene type in het andere over kan gaan.

Wij hebben getracht te onderzoeken of tussen beide typen duidelijke verschillen in antigene samenstelling bestaan. Ook deze vraag moet onbeantwoord blijven. Het is in dit verband van belang te vermelden, dat elke dierpassage een verandering in de antigene structuur van een virus kan geven. Kleine verschillen, die men met gevoelige methodes van onderzoek op het spoor komt, zijn zonder betekenis voor de door ons gestelde vraag.

Al deze problemen betreffende de virulentie en de antigene samenstelling doen ons wensen meer te weten te komen over het gedrag van het virus in de gastheer cel. Wij hebben een begin gemaakt met een quantitatief onderzoek in deze richting. Als onderwerp kozen wij de stofwisseling van desoxyribose nucleinezuur (D.R.N.). Deze stof immers (of polymeren ervan) moet als een essentieel bestanddeel van elk virus gezien worden. We waren in staat vast te stellen, dat vanaf een half uur of eerder, na het begin van de infectie, het D.R.N. gehalte van de cel een wijziging ondergaat. Wij vonden namelijk zowel in de kern als in het cytoplasma vanaf een half uur na de infectie minder D.R.N. Bij geïnactiveerd virus (inactief door verhitting gedurende een half uur op 56° C) konden wij aantonen dat het virus zich of wel tegen de kern aan legt, of wel in de kern wordt opgenomen. Wij vinden daar namelijk een verhoging van het D.R.N. van de kern. Merkwaardig is het verschil tussen dit geïnactiveerde virus en het onbehandelde virus; bij dit laatste vinden wij juist een vermindering van het D.R.N. gehalte van de nucleus. Op zichzelf is het belangrijk, dat het door verhitting gedurende een half uur op 56° C geïnactiveerde virus in de cel wordt opgenomen.

Naast het onderzoek over de stofwisseling van het D.R.N. in de bronchiaalepitheelcellen van de muizenlong na infectie met influenza virus (W.S.) hebben wij ook het verloop van de infectie in de muizenlong op de voet gevolgd met infectietiter en antigeen-titer bepalingen. In grote lijnen kunnen wij de resultaten van Hoyle, die dezelfde soort onderzoekingen op eieren uitvoerde, bevestigen. Wij vinden echter in tegenstelling tot Hoyle reeds twee tot twee en een half uur na de infectie een eerste stijging in de hoeveelheid virus die de cellen bevatten; n.l. én voor wat betreft de infectietiter bepalingen én voor wat betreft de antigeen titraties. Men zou deze aanvankelijke

stijging, die later weer door een daling gevolgd wordt, kunnen verklaren door aan te nemen, dat de infectiecyclus van het virus niet 4 uur, zoals Hoyle aanneemt, maar slechts twee uur duurt. Oorspronkelijk waren wij geneigd dit voor mogelijk te houden. Het onderzoek naar het D.R.N. gehalte van de cel heeft ons echter argumenten gegeven die daar tegen pleiten: 1e Direct na de infectie vinden wij de D.R.N. gehaltes van de kernen van bronchiaalepitheelcellen verminderd ten opzichte van controles; pas 4 uur na de infectie vinden wij opnieuw een dergelijke verlaging van het D.R.N. gehalte van de kernen. 2e Anderhalf uur na de infectie zijn de D.R.N. gehaltes van de kernen verhoogd, dit verschijnsel herhaalt zich pas drie tot drie en een half uur later. 3e Virus, dat door verhitten op 56° C ongeschikt is gemaakt voor de reproductie, wordt opgenomen in de bronchiaalepitheelcellen. Het bevindt zich daar oorspronkelijk tegen de kern aan of wordt in de kern opgenomen. Twee uur tot twee en een half uur na de infectie laat deze verbinding los en verschijnt het virus in het cytoplasma. Er is reden om aan te nemen, dat het virus op dat moment uit de cel gestoten wordt. Waarschijnlijk wordt virus, dat hoewel het in staat is zich te vermeerderen (of vermeerderd te worden), maar dat om de een of andere reden niet aan het reproductieproces deel neemt, op dezelfde wijze door de cel uitgestoten. Wij kunnen ons voorstellen dat zulke virusdeeltjes cellen binnen dringen waar te voren reeds een ander deeltje het reproductieproces in gang heeft gezet, die bezet zijn door inactief virus of dat ze cellen binnendringen, die niet voor de vermeerdering van het virus geschikt zijn.

Na het beëindigen van het reproductieproces (4 uur) komen enorme grote hoeveelheden virus vrij. De infectietiter (I.D. 50) van dit virus blijkt soms boven 10^{-10} voor eieren te stijgen. Tot nog toe waren wij bij muizenlong-suspensies en zelfs ook niet bij allantoisvocht zulke hoge I.D. 50 titers voor eieren tegen gekomen. Een verklaring menen wij te moeten zoeken in de omstandigheid dat het virus binnen 5 minuten na het doden van de muis op eieren geënt wordt. Uit andere proeven is reeds gebleken, dat in de eerste uren na het oogsten de I.D. 50 snel terugloopt. Als gevolg hiervan kunnen wij aannemen, dat onze virussuspensies vrij grote hoeveelheden inactief virus moeten bevatten en onze entingen dus meestal met een mengsel van veel inactief virus en weinig reproductief virus worden verricht.

In overeenstemming met de ontwikkeling van de T bacteriophaag in Escherichia coli kunnen wij bij de reproductie van het influenzavirus in de bronchiaalepitheelcel van de muizenlong verschillende ontwikkelingsphasen herkennen. In de eerste phase dringt het virus de cel binnen en wordt het aan de kern gebonden of misschien wel

daarin opgenomen. Of het op deze weg, juist zoals dit bij de bacteriophaag het geval is, een gedeelte van zijn samenstellende elementen verliest kan nog niet met zekerheid uitgemaakt worden. Er bestaan echter wel aanwijzingen in deze richting. Het virus immers verliest, ook als het niet aan het reproductieproces deelneemt, gedurende enige tijd het vermogen nieuwe cellen te infecteren. Deze eigenschap is echter ook voor andere uitlegging vatbaar. In de tweede phase wordt het D.R.N. van de kern afgebroken. In de daaropvolgende derde phase wordt weer D.R.N. in de kern opgebouwd, waarschijnlijk nu soortvreemd voor de cel, het virus-D.R.N. Na twee en een half à drie uur is een vierde phase te constateren. Dan zien wij een vermeerdering van de D.R.N. concentratie van het cytoplasma. Mischien is dit D.R.N. afkomstig van virus, dat niet aan het reproductieproces heeft deelgenomen en dat op dit moment loslaat van de kern en uit de cel wordt gestoten. In de laatste phase wordt ten slotte het „volwassen virus" gevormd. Het gehele proces is binnen vier uur afgelopen, na vier uur is de volgende eerste phase alweer voorbij en is de tweede phase van de ontwikkelingscyclus aan de gang.

Door vergelijking van de hier boven vermelde uitkomsten van het onderzoek, zoals wij dit met de influenzastam W.S. uitvoerden, met resultaten van onderzoekingen met andere stammen, hopen wij in staat te zijn gegevens te verzamelen die voor het virulentie vraagstuk van belang zijn. In ieder geval moet het mogelijk zijn op deze wijze een aantal eigenschappen van het influenzavirus nader te bestuderen. Bovendien kunnen wij behalve kernzuren ook andere substanties tijdens het ontwikkelingsproces van het influenzavirus vervolgen. Tenslotte kunnen wij de door ons beschreven methodes van onderzoek ook op andere virales toepassen.

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